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# THE CORROSION OF CONCRETE

## 1. THE ISOLATION OF A SPECIES OF BACTERIUM ASSOCIATED WITH THE CORROSION OF CONCRETE EXPOSED TO ATMOSPHERES CONTAINING HYDROGEN SULPHIDE.

by C. D. PARKER[1,2]

(From the Melbourne and Metropolitan Board of Works.)

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The internal surface of concrete sewers is frequently subject to rapid corrosion, leading in some cases to complete disintegration. This particular form of corrosion occurs only where the sewage and the sewer atmosphere contain an appreciable quantity of hydrogen sulphide ($H_2S$). The corrosion occurs only above water level, the point of most rapid corrosion being just above the daily high water level.

Where the corrosion process has become well established and is proceeding rapidly the cement constituents of the concrete are completely broken down leaving the coarse aggregate embedded in a putty-like mass. The cement constituents are converted to sulphates and a high concentration of free sulphuric acid (1–5 p.c.) is frequently found in the moisture associated with the corrosion products.

This form of corrosion is of widespread occurrence. It has been observed at Cairo (Hammerton, 1939), Cape Town (Morris, 1940), Los Angeles (Studley, 1939), and at Orange County and El Centro County, Calif. (Goudey, 1929). It also occurs in the sewerage systems of most Australian capital cities and many large country towns (private communications). In Melbourne, marked corrosion is present in the main outfall sewer, and at some other points in the main sewerage system and the south-eastern system.

Numerous authors, e.g., Olmstead and Hamlin (1900), Barr and Buchanan (1912), Mahlie (1934), Baumgartner (1934), Bunker (1936), and Lea and Desch (1936), have attributed the corrosion to the presence of $H_2S$. Apart from the observed association of $H_2S$ with the process, and the detection of sulphates and free sulphuric acid in the corrosion products, there is little published evidence to indicate the method of conversion of the $H_2S$ to sulphates and free acid. Bunker (1936) suggests that the $H_2S$ is oxidized non-biologically and Lea and Desch (1936) suggest a possible series of chemical reactions, both without experimental evidence.

The Engineering Staff of the Melbourne and Metropolitan Board of Works in conjunction with the Board's Consultant Chemist (Mr. V. G. Anderson), has obtained over a number of years considerable information (unpublished) concerning the practical and chemical aspects of the problem.

Thus, the development of free sulphuric acid and the production of sulphates of calcium, iron and aluminium from the cement, have been established. Free sulphur has also been found. Based on this work the view has been held that (1) the direct cause of the corrosion is the production of sulphuric acid but that (2) while the conversion of $H_2S$ to free sulphur, and the reaction between sulphuric acid and the cement constituents can be explained on purely chemical grounds, it

[1] Work carried out by the author as part of a comprehensive investigation into the problem of the corrosion of sewer structures by hydrogen sulphide, which is being carried out by the Research Staff of the Sewerage Branch of the Melbourne and Metropolitan Board of Works.

[2] Senior Chemist and Bacteriologist, Research Laboratories.

is difficult to explain the production, non-biologically, of sulphuric acid from sulphur.

The inadequacy of this explanation, the behaviour in the present investigation of test specimens exposed to sewer atmospheres, and the high acidity developed suggested the possible association of a biological agent with the process.

This paper describes the isolation of a species of bacterium from the corrosion products and its physiological and biochemical properties.

## EXPERIMENTAL.

*Isolation.*

The following medium was used. Free sulphur was used as a source of sulphur owing to the difficulty in maintaining an $H_2S$ atmosphere for incubation and the probability that any organism capable of oxidizing $H_2S$ would also oxidize free sulphur: potassium dihydrogen phosphate, 3 gm.; magnesium chloride (anhyd.), 0·5 gm.; calcium chloride (anhyd.), 0·25 gm.; ammonium chloride, 0·2 gm.; precipitated sulphur, 10 gm.; distilled water, 1,000 ml.

The constituents, apart from the sulphur, were dissolved in the distilled water and 25 ml. quantities distributed in 100 ml. Erlenmeyer flasks. These were then autoclaved. The sulphur was put up in 0·25 gm. amounts in test tubes and sterilized by three successive steamings. One test tube of sulphur was added to each flask of medium which was then ready for inoculation. The pH of the medium was 4·5–5·0.

About 3 gm. of moist corroded concrete were inoculated into a flask of this medium and incubated at 30° C. After about 14 days the liquid became turbid and had developed a marked acidity due to the presence of sulphuric acid. A hanging drop preparation revealed the presence of large numbers of small non-motile rods in the liquid.

The growth in the initial flask was sub-cultured, twice in liquid medium, and then plated on to a solid medium similar in constitution to that used by Waksman (1922) in the isolation of *Thiobacillus thiooxidans*. It consisted of: $Na_2S_2O_3$, 5·0 gm; $KH_2PO_4$, 3·0 gm.; $NH_4Cl$, 0·1 gm.; $MgCl_2$, 0·1 gm.; $CaCl_2$, 0·25 gm.; agar, 20 gm.; distilled water, 1,000 ml.

The medium was sterilized by steaming on three successive days. On plating from the second liquid sub-culture, colonies developed in 5–7 days. The colonies and the organisms comprising them were all identical in appearance. From these plates single colony cultures were obtained. Uniformity of morphology and colony form, and the absence of growth on nutrient agar, proved the purity of the culture isolated.

In this way cultures were isolated from specimens of corroded concrete from five different localities, and designated A, B, C, D, E.

*Concentration in Corrosion Products.*

To obtain some idea of the numbers of these acid-forming organisms present in specimens of corroded concrete, dilutions were made of a water extract of one gm. of the corroded material and one ml. quantities inoculated into the liquid medium. Typical cultures were obtained from 50,000–5,000,000 fold dilutions of the original concrete specimen indicating the presence of at least 50,000–5,000,000 organisms per gm. of corroded concrete.

*Morphology.*

All five strains are similar. They are slender rods 1·5–2·0$\mu$ long by 0·5$\mu$ wide, with straight sides and square ends. They stain irregularly, showing deeply stained granules in a poorly stained slender rod. The appearance on staining with 0·5 p.c. methylene blue is somewhat reminiscent of *C. diphtheriae*. They are Gram negative. Young cultures (2–5 days) show a few motile forms but in older cultures no motility is observed. None of the strains forms spores and liquid cultures are killed by exposure to a temperature of 60° C for 10–20 minutes.

*Cultural Characteristics.*

In liquid media.

(1) Nutrient broth: no growth occurs even when the pH is adjusted to 4·0.

(2) Medium for isolation: all strains produce uniform turbidity usually in from 36–72 hours which increases in density until the fifth day and then remains constant. The sulphur, which floats on the surface of the liquid in the uninoculated medium, falls to the bottom some days after turbidity appears.

Strain D also produces a flocculent precipitate after prolonged incubation. None of the strains produces a surface pellicle. The autoagglutination of cultures of *Thiobacillus thiooxidans* when filtered free from sulphur through a filter paper described by Waksman and Joffe (1922) is not shown by any of the strains, even after several months standing.

(3) Thiosulphate medium: all strains develop in a medium similar to that used for isolation, in which the free sulphur is replaced by thiosulphate. They all produce a uniform turbidity and deposit a variable amount of free sulphur.

On solid media.

(1) Thiosulphate agar (as used for isolation). The five strains show marked differences in their colony form on thiosulphate agar plates.

Strain A. After 5–10 days forms minute circular water clear colonies 0·2 mm. diameter. No change occurs on further incubation. They do not produce confluent growth. There is no cloudiness produced in the medium.

Strain B. Produces minute water clear circular colonies in four days which grow to 0·5 mm. diameter after nine days. Most of the colonies coalesce to form a confluent growth which has a white etched-glass appearance. The few single colonies present at nine days are convex, circular, with an entire edge and are butyrous in consistency. They are water clear in appearance. The medium near the line of growth first becomes cloudy, presumably due to liberation of sulphur, and then clears due to its subsequent oxidation.

Strain C. Minute water-clear colonies appear in two days which grow to 0·75 mm. diameter in nine days and become dirty yellowish-white in colour. They are convex, circular, with an entire edge and are of butyrous consistency. There is no tendency to form confluent growth. The medium becomes cloudy and then clears, as with Strain B.

Strain D. Minute pin point water-clear colonies appear in two days which grow to 0·5 mm. in diameter in nine days and become cream in colour although they do not become as yellow as those of the C strain. Colonies are circular, convex with an entire edge and of butyrous consistency. There is no tendency to form confluent growth. This strain produces a slight cloudiness and clearing of the medium.

Strain E. Roughly circular colonies 0·5 mm. in diameter appear in two days. Radiating hair-like outgrowths come from a central raised point. The surface is rough and the edge is irregular. The colonies have an etched glass appearance. They grow to a size of 1–2 mm. in diameter in 9 days, still retaining their original appearance. There is a considerable tendency to form a confluent growth. The growth is butyrous in consistency. There is considerable cloudiness and subsequent clearing of the medium.

(2) Nutrient agar. No growth occurs even when the pH is adjusted to 5·0.

*Biochemical Properties.*

*Aerobiosis.* All strains are aerobic.

*Autotrophism.* None of the strains grow on nutrient agar. If inoculated into the above sulphur liquid medium and incubated in a $CO_2$-free atmosphere no growth occurs. The replacement of $CO_2$ by carbonates, bicarbonates or glucose does not permit growth. The five strains are therefore obligatory autotrophs utilizing $CO_2$ only for the synthesis of organic carbon.

To determine whether the presence of organic carbon influences their ability to oxidise sulphur to $H_2SO_4$, a medium was prepared as for their isolation and to it were added three different concentrations each of glucose, glycerol and sodium lactate. The flasks were incubated at 30° C. and the activity of the various strains was assessed by the amount of sulphuric acid produced. This was determined by titration of 2 ml. of the medium with N/20 NaOH after varying periods of incubation. The acidities developed in 30 days by the five strains, in the presence of the various concentrations of the three different sources of organic carbon, are shown in Table 1.

Glucose slightly increases the activity of strains B and C in low concentration, but retards the activity of strains A, D and E. In concentrations of 1 p.c. or higher, it retards the activity of all strains, and practically prevents the activity of strains A and E altogether.

Glycerol, in all concentrations, slightly reduces acid formation by all strains, although a concentration of 5 p.c. does not completely inhibit the activity of any strain.

Sodium lactate slightly inhibits acid formation by all strains except strain B in a concentration of 50 p.p.m. Concentrations, of 1 p.c. or more, completely inhibit the activity of all strains.

In general, organic carbon as glucose, glycerol and lactate has little influence in low concentrations, in some cases showing a slight stimulative effect and in others being slightly inhibitive. Higher concentrations reduce acid formation, in some cases resulting in complete inhibition of growth. The five strains do not show any marked difference in their reaction to the addition of organic carbon.

*Compounds of sulphur oxidised.* The following forms of sulphur were used to ascertain what compounds of sulphur could be utilized for growth and acid formation, i.e. elementary sulphur, thiosulphate, sulphite, sodium sulphide and $H_2S$. In this and the following biochemical studies, the liquid medium used for isolation was adopted as a basic medium and varied

according to the constituent to be studied. In this case the medium was made up with all constituents other than the elementary sulphur and the test media prepared by the addition of 10 gm./litre of sulphur added as sodium thiosulphate, sodium sulphide, sodium sulphite and elementary sulphur. In the case of the $H_2S$ an atmosphere of 1 p.c. $H_2S$ (generated in a Kipp's apparatus) was passed over the surface of the inoculated medium.

TABLE 1.

*Effect of organic carbon compounds on the ability of the five strains to produce sulphuric acid.*

| Nature of organic compound. | Strain. | Amount of acid produced (ml. N/20 NaOH = 2 ml. of media) after 30 days incubation. Concentration of organic compound added. None. | 50 p.p.m. | 1 p.c. | 5 p.c. |
|---|---|---|---|---|---|
| Glucose | A | 18·15 | 13·25 | 0·75 | 0·75 |
| | B | 19·85 | 21·85 | 12·15 | 9·25 |
| | C | 17·25 | 20·60 | 5·70 | 10·25 |
| | D | 14·90 | 10·15 | 6·75 | 7·40 |
| | E | 16·10 | 11·95 | 0·70 | 1·00 |
| Glycerol | A | 18·15 | 14·35 | 12·70 | 12·65 |
| | B | 19·85 | 15·95 | 13·35 | 12·90 |
| | C | 17·25 | 17·10 | 13·80 | 11·15 |
| | D | 14·90 | 8·75 | 9·95 | 11·65 |
| | E | 16·10 | 14·15 | 5·75 | 5·45 |
| Sodium lactate | A | 29·45 | 27·25 | 0·35 | 0·35 |
| | B | 26·50 | 29·30 | 0·40 | 0·70 |
| | C | 30·35 | 23·30 | 0·35 | 0·60 |
| | D | 25·70 | 20·20 | 0·45 | 0·50 |
| | E | 20·55 | 17·20 | 0 | 0·20 |
| Glucose | Sterile control | 0 | | | 0·35 |
| Glycerol | " " | 0 | | | 0·45 |
| Sodium lactate | " " | 0 | | | 0·25 |

The basic medium (less the elementary sulphur) was prepared and 100 ml. amounts distributed into four sets of 300 ml. Erlenmeyer flasks. These were plugged with cotton wool and autoclaved. Strong solutions of sodium thiosulphate, sodium sulphide and sodium sulphite were prepared, sterilized by Seitz filtration and sufficient of the sterile solutions added to the flasks in three of the sets to produce a final concentration of 10 gm./litre of sulphur. The free sulphur was sterilized in 1 gm. lots in test tubes, by steaming and the contents of one test tube added to each flask of the fourth set. The medium in which the sulphur was to be available as $H_2S$ was distributed in 750 ml. Erlenmeyer flasks, fitted with rubber stoppers and inlet and outlet tubes. The inlet and outlet tubes were plugged with cotton wool and the flasks autoclaved . The flasks were then connected in series and finally connected to the $H_2S$ supply. The pH of all media was adjusted to 5–6 and each of the five strains was inoculated into one flask of medium containing each of the different sources of sulphur.

The four sets of flasks, other than those supplied with $H_2S$, were incubated at 30° C. The set supplied with $H_2S$ was incubated at room temperature (about 23° C.). The ability to utilize the various forms of sulphur was determined by titration of the acidity developed and gravimetric determination of sulphate formation by the standard barium chloride method. In the case of the thiosulphate medium, destruction of thiosulphate was followed by iodine titration. The changes in the sulphur compounds brought about by the five strains after incubation for 20 and 40 days are shown in Table 2.

The negative values for the thiosulphate destroyed by strain E and the sterile control are explained by the evaporation and consequent concentration of the media after prolonged incubation. This fact renders the exact quantitative significance of the results somewhat uncertain. However, the results permit of the qualitative and roughly quantitative determinations of the ability of the five strains to utilize various sulphur compounds.

All five strains are similar, in that growth occurred with free sulphur and thiosulphate as the source of sulphur, while no growth occurred when the source of sulphur was sulphite, sodium sulphide or $H_2S$. There appears to be no marked difference in their ability to oxidize sulphur to sulphuric acid, while the substantial agreement between the sulphate formed as determined by

TABLE 2.

*The ability of the five strains to utilize various forms of sulphur.*

Concentration of sulphur compounds (as p.p.m. of sulphur).

| Source of sulphur. | Strain. | Initial concentration. (1) $SO_4$.S Grav. | (2) $SO_4$.S Titn. | (3) Original S. compounds destroyed. | After 20 days. (1) $SO_4$.S Grav. | (2) $SO_4$.S Titn. | (3) Original S. compounds destroyed. | (4) =(3)−(1) Free S. deposited. (?) | After 40 days. (1) $SO_4$.S Grav. | (2) $SO_4$.S Titn. | (3) Original S. compounds destroyed. | (4) =(3)−(1) Free S. deposited. (?) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Elementary sulphur | A | 22·0 | 0 | nil | 4,946 | 4,840 | n.d.* | | 11,470 | 11,760 | n.d. | |
| | B | „ | 0 | „ | 4,119 | 3,680 | „ | | 10,017 | 10,500 | „ | |
| | C | „ | 0 | „ | 3,800 | 3,640 | „ | | 10,401 | 10,840 | „ | |
| | D | „ | 0 | „ | 2,977 | 3,020 | „ | | 9,163 | 8,980 | „ | |
| | E | „ | 0 | „ | 58 | 40 | „ | | 5,838 | 6,000 | „ | |
| | Sterile control | „ | 0 | „ | 33 | 0 | „ | | 60 | 40 | „ | |
| Thio-sulphate | A | 324 | 0 | nil | 816 | 100 | 1,040 | 226 | 3,820 | 100 | 6,440 | 2,620 |
| | B | „ | 0 | „ | 5,572 | 860 | 9,840 | 4,268 | 7,609 | 1,340 | 10,320 | 2,721 |
| | C | „ | 0 | „ | 9,531 | 4,280 | 9,920 | 389 | 12,401 | 6,140 | 10,320 | — |
| | D | „ | 0 | „ | 4,350 | 120 | 8,960 | 4,610 | 6,805 | 620 | 10,160 | 3,355 |
| | E | „ | 0 | „ | 494 | 120 | 400 | — 94 | 799 | 140 | — 240 | — 1,039 |
| | Sterile control | „ | 0 | „ | 283 | 100 | — 1,360 | — 1,643 | 478 | 120 | — 5,760 | — 6,238 |
| | | | | | | | | | After 30 days. | | | |
| Sulphite | A | 3,158 | 0 | nil | 6,615 | 0 | n.d. | n.d. | 6,991 | 0 | n.d. | n.d. |
| | B | „ | 0 | „ | 6,772 | 0 | „ | „ | 7,442 | 0 | „ | „ |
| | C | „ | 0 | „ | 6,766 | 0 | „ | „ | 7,112 | 0 | „ | „ |
| | D | „ | 0 | „ | 6,483 | 0 | „ | „ | 7,368 | 0 | „ | „ |
| | E | „ | 0 | „ | 6,741 | 0 | „ | „ | 6,849 | 0 | „ | „ |
| | Sterile control | „ | 0 | „ | 6,752 | 0 | „ | „ | 7,296 | 0 | „ | „ |
| Sodium sulphide | A | 60 | 0 | nil | 77 | 0 | n.d. | n.d. | 71 | 0 | n.d. | n.d. |
| | B | „ | 0 | „ | 66 | 0 | „ | „ | 183 | 0 | „ | „ |
| | C | „ | 0 | „ | 88 | 0 | „ | „ | 49 | 0 | „ | „ |
| | D | „ | 0 | „ | 88 | 0 | „ | „ | 77 | 0 | „ | „ |
| | E | „ | 0 | „ | 80 | 0 | „ | „ | 88 | 0 | „ | „ |
| | Sterile control | „ | 0 | „ | 71 | 0 | „ | „ | 69 | 0 | „ | „ |
| Hydrogen sulphide | A | 22 | 0 | n.d. | | | | | 78 | 0 | | |
| | B | „ | 0 | „ | | | | | 39 | 0 | | |
| | C | „ | 0 | „ | | | | | 40 | 0 | | |
| | D | „ | 0 | „ | | | | | 33 | 0 | | |
| | E | „ | 0 | „ | | | | | 24 | 0 | | |
| | Sterile control | „ | 0 | „ | | | | | 36 | 0 | | |

* n.d.: not determined.

titration and by the gravimetric method, indicates that the whole of the sulphate formed from elementary sulphur is present as free sulphuric acid.

While all five strains are capable of growth when sulphur is supplied as thiosulphate, they differ considerably in their oxidation products. Strains A and E attack thiosulphate slowly with the production of small amounts of sulphur and little or no development of free acid. Strains B, C and D rapidly attack thiosulphate but whereas strains B and D produce little free acid and much free sulphur, strain C produces a considerable amount of free acid and little free sulphur.

*Ability to utilize various sources of nitrogen.* The ability to utilize various form of nitrogen for growth has been shown by Starkey (1925b) to be of differential importance amongst the sulphur bacteria. The five strains were accordingly tested for their ability to utilize ammonium, nitrite, nitrate and organic nitrogen as the sole source of nitrogen. The organic nitrogen was supplied either as asparagine or peptone.

TABLE 3.

*The ability of the five strains to utilize various forms of nitrogen.*

| | | Amount of acid produced (ml. N/20 NaOH = 2 ml. of media) in 20 days. Concentration of nitrogen (p.p.m.). | | |
|---|---|---|---|---|
| Source of nitrogen. | Strain. | 5 p.p.m. | 50 p.p.m. | 500 p.p.m. |
| Ammonium | A | 5·10 | 10·70 | 11·45 |
| | B | 4·60 | 9·25 | 8·65 |
| | C | 7·35 | 11·85 | n.d. |
| | D | 4·95 | 10·05 | n.d. |
| | E | 4·75 | 7·60 | n.d. |
| | Sterile control | | | 0 |
| Nitrite | A | 0·15 | 0 | 0 |
| | B | 0·15 | 0 | 0 |
| | C | 0·10 | 0 | n.d. |
| | D | 0·10 | 0 | ,, |
| | E | 0·10 | 0 | ,, |
| | Sterile control | | | 0·05 |
| Nitrate | A | 4·60 | 11·20 | 9·60 |
| | B | 5·65 | 7·70 | 4·15 |
| | C | 6·80 | 14·15 | n.d. |
| | D | 4·70 | 10·90 | ,, |
| | E | 4·30 | 6·45 | ,, |
| | Sterile control | | | 0 |
| Asparagine | A | 0 | 3·75 | 0·85 |
| | B | 0 | 13·80 | 0·25 |
| | C | 4·45 | 0 | n.d. |
| | D | 3·15 | 4·10 | ,, |
| | E | 0·10 | 0 | ,, |
| | Sterile control | | | 0·15 |
| Peptone | A | 3·80 | 8·80 | 0 |
| | B | 4·65 | 9·75 | 0 |
| | C | 5·70 | 6·95 | n.d. |
| | D | 3·50 | 6·85 | ,, |
| | E | 3·05 | 5·45 | ,, |
| | Sterile control | | | 0 |
| Ammonium 50 p.p.m. + nitrite 50 p.p.m. | A | | 0·10 | |
| Ammonium 50 p.p.m. + nitrate 50 p.p.m. | A | | 10·20 | |
| Ammonium 50 p.p.m. + asparagine 50 p.p.m. | A | | 12·70 | |
| Ammonium 50 p.p.m. + peptone 50 p.p.m. | A | | 11·10 | |

The basic medium was prepared as above but without the ammonium chloride. Nitrogen was then added in three concentrations (5, 50 and 500 p.p.m.) in the form of ammonium chloride, sodium nitrite, sodium nitrate, asparagine and peptone. The fifteen media so prepared were distributed into 300 ml. Erlenmeyer flasks in 100 ml. quantities. The ammonium chloride and sodium nitrite and nitrate media were sterilized by autoclaving, after distribution into 300 ml. flasks. The asparagine and peptone media were sterilized in bulk by Seitz filtration and then distributed under aseptic conditions. This was necessary as autoclaved media containing asparagine and peptone were found to contain 5–10 p.p.m. of ammonium nitrogen. The media prepared by Seitz filtration were found to contain less than 1 p.p.m. of ammonium nitrogen.

Media were also prepared containing 50 p.p.m. of ammonium nitrogen plus 50 p.p.m. of nitrogen as nitrite, nitrate, asparagine and peptone, respectively.

The five strains were inoculated into media containing the three concentrations of the five single sources of nitrogen and incubated at 30° C. The inoculum used was 0·2 ml. of liquid culture which introduced less than 0·1 p.p.m. of ammonium nitrogen into the various media. The media, with ammonium plus another source of nitrogen, were inoculated with strain A only. The ability of the strains to utilize the different forms of nitrogen was determined by titration of the acidity developed after varying periods of incubation. The acidity developed in 20 days with the different nitrogen sources is shown in Table 3.

The five strains are similar in that they can utilize ammonium and nitrate nitrogen equally well. Peptone nitrogen results in a slight decrease in the rate of acid formation and nitrite cannot be used by any strain. The results with asparagine are somewhat irregular, strains A, B and D producing acid, while strains C and E are unable to do so at a nitrogen concentration of 50 p.p.m. A concentration of approximately 50 p.p.m. of nitrogen appears to be optimal for acid formation with all strains. Nitrite appears to be toxic as no acid formation occurs in the presence of nitrite, even when ammonium nitrogen is available. The presence of nitrate, asparagine or peptone nitrogen, in addition to ammonium nitrogen, does not affect the rate of acid formation.

*Effect of concentration of nitrogen on rate of acid formation.* The results in Table 3 suggest that the concentration of nitrogen under some circumstances may be a limiting factor in acid formation. Media were therefore prepared, using the basic medium minus the ammonium chloride. Ammonium chloride was then added to give a range of concentrations of nitrogen. The first series covered the range 0–100 p.p.m. of nitrogen and four strains were put up on this range of nitrogen concentrations. The media were distributed as before into 300 ml. Erlenmeyer flasks and inoculated with 0·05 ml. of culture. The acidity developed after 20 and 50 days in media containing the above range of nitrogen concentrations, is shown in Table 4.

TABLE 4.

*Effect of concentration of ammonia nitrogen (0–100 p.p.m.) on rate of acid formation.*

Amount of acid produced (ml. N/20 NaOH = 2 ml. media).

| Concentration of nitrogen p.p.m. | Strain. A. After 20 days. | A. After 50 days. | B. After 20 days. | B. After 50 days. | D. After 20 days. | D. After 50 days. | E. After 20 days. | E. After 50 days. |
|---|---|---|---|---|---|---|---|---|
| 0 | 0·60 | 1·70 | 0·80 | 2·45 | 0·70 | 2·00 | 0·45 | 1·40 |
| 1 | 1·65 | 4·65 | 1·80 | 4·10 | 1·65 | 3·45 | 1·25 | 2·80 |
| 5 | 3·70 | 6·85 | 4·55 | 9·90 | 4·10 | 8·70 | 2·55 | 6·05 |
| 10 | 7·30 | 12·30 | 8·30 | 15·20 | 6·60 | 13·95 | 4·45 | 10·75 |
| 50 | 9·10 | 29·45 | 15·55 | 44·75 | 8·90 | 30·15 | 8·55 | 32·45 |
| 100 | 7·15 | 23·85 | 16·90 | 40·95 | 9·50 | 27·00 | 7·60 | 26·75 |
| Sterile control | 0 | 0·05 | 0 | 0·05 | 0 | 0·05 | 0 | 0·05 |

In order to obtain information with very small concentrations of nitrogen, media were made up to contain a range of ·005–2·0 p.p.m. of nitrogen. These were inoculated with growth from the flasks containing 1·0 p.p.m. of nitrogen in the experiment tabulated in Table 4. This inoculation introduced ·005 p.p.m. of nitrogen into the medium. The acid developed on incubation is shown in Table 5.

Table 4 indicates that the rate of acid formation increases with the nitrogen concentration up to about 50 p.p.m., above this concentration there appears to be a slight inhibition. All four strains behave similarly over this range of nitrogen concentration.

The small concentrations of nitrogen, shown in Table 5, limit the rate of acid formation, but slight acid formation, compared with the sterile control, does occur, even with ·005 p.p.m. of nitrogen.

TABLE 5.

*Effect of concentration of ammonium nitrogen (·005–2·0 p.p.m.) on rate of acid formation.*

| Nitrogen concentration p.p.m. | Amount of acid produced. Strain A. | |
|---|---|---|
| | After 20 days. | After 50 days. |
| ·005 | 0·25 | 0·80 |
| ·01 | 0·25 | 0·85 |
| ·05 | 0·35 | 0·80 |
| ·10 | 0·33 | 1·15 |
| ·20 | 0·37 | 0·95 |
| ·50 | 0·75 | 1·25 |
| 1·00 | 1·02 | 1·90 |
| 2·00 | 1·38 | 3·15 |
| Sterile control | 0 | 0·10 |

*Phosphate requirements.* It has recently been shown by Vogler and Umbreit (1942) that phosphate is an essential link between the sulphur oxidation and carbon dioxide assimilation processes of the acid-forming type of sulphur bacteria, but no information is available as to the minimum concentration of phosphate necessary for optimum acid formation. As it is possible that phosphate supply might be a limiting factor in the formation of acid on corroding concrete, the rate of acid formation was determined in media of variable phosphate content. The basic medium (less the $KH_2PO_4$) was prepared as before and phosphate added to give concentrations of ·0005, ·005, ·01, ·05, ·10, ·20, ·50, 1·0, 2·0, 10·0, 100 and 682 p.p.m. of phosphorus as $KH_2PO_4$. The phosphate content of the remaining constituents of the basic medium was determined by the analysis of sufficient of the mineral salts necessary to prepare 10 litres of medium. Phosphate determination was by the standard gravimetric method of precipitation as ammonium phosphomolybdate. This analysis indicated that the remainder of the salts, apart from the phosphate deliberately added, would contribute ·0005 p.p.m. of phosphate phosphorus. This was accordingly allowed for in preparing the above media. The pH of each medium was adjusted to 4·5, the media autoclaved and inoculated with 0·1 ml. of strains A and B. The cultures used for inoculation were specially prepared in media of low phosphate content. A single loopful (·02 ml.) of culture in the ordinary basic medium was inoculated into 25 ml. of the basic medium without phosphate. The consequent phosphate concentration in the resultant culture flask was approximately 0·5 p.p.m. of phosphorus. Growth occurred in this culture flask with both strains A and B and this culture was used to inoculate the media containing the varying amounts of phosphorus, there being ·0005 p.p.m. of phosphorus added in the inoculum. The acidity developed after 30 days incubation is shown in Table 6.

TABLE 6.

*Effect of phosphate concentration on acid formation.*

| Concentration p.p.m. P. | Amount of acid produced after 30 days. | |
|---|---|---|
| | Strain A. | Strain B. |
| ·0005 | 0·35 | 0·45 |
| ·005 | 1·65 | 0·20 |
| ·01 | 1·80 | 0·85 |
| ·05 | 2·95 | 2·75 |
| ·10 | 5·20 | 4·80 |
| ·20 | 9·50 | 7·85 |
| ·50 | 19·30 | 16·55 |
| 1·0 | 21·80 | 18·75 |
| 2·0 | 23·35 | 20·00 |
| 10·0 | 20·55 | 20·65 |
| 100 | 21·65 | 15·90 |
| 682 | 21·40 | 19·85 |
| Sterile control | 0·05 | 0·05 |

If the amount of acid formed is plotted, on squared paper, against the phosphorus concentration, it is found to be directly proportional to the phosphate content up to a concentration of 0·5 p.p.m. of phosphorus. Little change in the rate of acid formation occurs when the phosphorus concentration is raised above 0·5 p.p.m.

*Effect of pH of medium on ability to grow and develop acidity.* A freshly poured concrete structure is strongly alkaline in reaction owing to the calcium hydroxide liberated on hydration

of the cement compounds. Exposed to atmospheric conditions, the concrete remains alkaline, despite the carbonation of the lime. In view of the association of these organisms with corroded concrete, the range of the pH of the medium, over which they can develop, is of some significance.

Quantities of the basic medium were adjusted at pH values over a range from 2 to 8. Each of the five strains was inoculated into media at each of the different pH values. All strains developed turbidity and acidity, most rapidly, at a pH of 4·0, and could grow and develop acidity, at pH of 6·0 or less. None was capable of growth at pH 6·5 or higher.

*Acid and alkali tolerance.* Exposure of the five strains in phosphate buffer solutions of pH 6, 7, 8, 9, 10 for 24 hours resulted in the destruction of all strains at pH greater than 6·0. All strains could be sub-cultured from the buffer solution adjusted to pH 6·0. The maximum concentration of acid, which these organisms can develop and withstand, has not been ascertained precisely. However, cultures of strains B and D, incubated for 80 days developed an acidity equal to 10 p.c. of sulphuric acid, while the other three strains developed acidities of 8 p.c. sulphuric acid and were still actively producing acid. Subcultures revealed that the organisms were still viable.

*Temperature.* Strain A was inoculated into the basic medium and flasks incubated at temperatures of 4°, 10–12°, 20°, 30°, 37° C. The acidity developed after 50 days incubation is shown in Table 7.

TABLE 7.

*Effect of temperature on acid formation.*

| Temperature ° C. | Amount of acid produced after 50 days. |
|---|---|
| 4° | 0 |
| 10–12° | 7·33 |
| 20° | 9·05 |
| 30° | 34·42 |
| 37° | 26·18 |

These organisms have a temperature optimum of about 30° C., considerable acid is still formed at 37° C. but at temperatures below 30° C. the rate of acid formation rapidly decreases until at 4° C. the activity is negligible.

*Taxonomy.*

The five strains here described obviously belong to the group of sulphur oxidizing bacteria which has been reviewed by Waksman (1932), Bunker (1936) and Stephenson (1939).

They approximate most closely to organisms of the sub-group which produce and withstand high concentrations of sulphuric acid. The only species of this sub-group described in sufficient detail for comparative purposes is *Thiobacillus thiooxidans* (Waksman and Joffe, 1922; Waksman and Starkey, 1922; Starkey, 1925a and b).

They resemble *Th. thiooxidans*, in that they are strictly autotrophic, they oxidize sulphur to free sulphuric acid, they do not utilize $H_2S$ and they only develop in acid media.

They differ from *Thiobacillus thiooxidans* in the following respects:

1. Morphologically, they are slender rods 1·5–2·0μ by 0·5μ while *Th. thiooxidans* is 0·75–1·0μ by 0·5μ.
2. Colony form on thiosulphate agar varies from strain to strain and for most strains is different from that of *Th. thiooxidans*.
3. They are definitely inhibited, while *Th. thiooxidans* is stimulated, by organic carbon compounds.
4. They utilize nitrate as well as ammonium nitrogen while *Th. thiooxidans* can only utilize ammonium nitrogen.
5. They precipitate sulphur from thiosulphate in some cases producing little or no free acidity. *Th. thiooxidans* converts thiosulphate directly to sulphuric acid.
6. These strains are associated with corroding concrete sewers while *Th. thiooxidans* has been isolated from a compost of soil, sulphur and rock phosphate.

The five strains therefore are considered to differ sufficiently from *Th. thiooxidans* to justify their description as a separate species.

COMMENT.

The name *Thiobacillus concretivorus* is proposed for this species of bacterium isolated from corroded concrete and the five strains studied are considered to be five separate strains belonging to the one species.

The activity of this species of bacterium would explain the production of free sulphuric acid in the corrosion process, provided free sulphur or some utilizable form of sulphur is first formed from the $H_2S$. The experimental proof of the action of the organism will be described in a further paper.

## SUMMARY.

Five strains of sulphuric-acid-forming bacteria have been isolated in pure culture from corroded concrete exposed to atmospheres containing $H_2S$.

The morphological cultural and biochemical properties of these five strains have been described and appear to warrant their differentiation as strains of a new species of sulphur bacteria, which has provisionally been named *Thiobacillus concretivorus*.

*Acknowledgments.* I wish to thank the Engineer of Sewerage, Melbourne and Metropolitan Board of Works (Mr. E. F. Borrie, M.C., M.C.E., M.I.E.Aust.), at whose direction the work was carried out, for his interest in the investigation and for his permission to publish this paper. I also wish to thank Miss S. E. Gorham for her assistance in carrying out the work.

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# THE CORROSION OF CONCRETE

## 2. THE FUNCTION OF *THIOBACILLUS CONCRETIVORUS* (NOV. SPEC.) IN THE CORROSION OF CONCRETE EXPOSED TO ATMOSPHERES CONTAINING HYDROGEN SULPHIDE.

by C. D. PARKER[1,2]

(From the Melbourne and Metropolitan Board of Works).

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The widespread occurrence of corrosion of concrete structures carrying sewage, has been noted in a previous paper (Parker, 1945). The visible effect of this type of corrosion under South African conditions has been described in some detail by Morris (1940) and in a discussion of Morris's paper by Woolley (1940). The form of corrosion under Australian conditions is apparently similar, the chief characteristics being as follows (1) it is associated with the presence of hydrogen sulphide in the sewer atmosphere; (2 )it occurs above water level where the concrete surface is in contact with the gaseous $H_2S$ emitted from the sewage; (3) the ultimate result of corrosion is the conversion of the concrete structure to a putty-like mass in which the particles of aggregate are embedded; (4) test specimens placed in the sewer show a definite lag period in the development of corrosion. Once corrosion commences it proceeds rapidly.

Concrete is a mixture of cement, fine aggregate (usually quartz sand), coarse aggregate (basalt is frequently used in Melbourne) and water. The chemically active constituents are the cement and water, the properties of concrete being dependent on the reaction which occurs between them. In mixing concrete, hydration of the cement constituents, tricalcium aluminate, tricalcium silicate, dicalcium silicate and tetra calcium aluminoferrite, occurs. It is these hydration compounds which are responsible for the properties of concrete. The aggregate may be considered to be inert material bound together by the hydrated cement compounds. During hydration, calcium hydroxide is liberated and the set concrete is highly alkaline (pH 11–12·5). On exposure to the atmosphere, carbonation occurs until the calcium hydroxide at the exposed surface is completely converted to calcium carbonate (saturated $CaCO_3$ solution has pH 10·2). In the presence of moisture some of the carbonate is dissolved as bicarbonate and the equilibrium, solid calcium carbonate-saturated solution of calcium bicarbonate-atmospheric $CO_2$, is set up. The bicarbonate solution under such conditions has a pH of 8·4. Concrete exposed to the atmosphere becomes chemically stable at this stage.

Concrete exposed to corrosive sewer atmospheres undergoes further changes producing a continued shift towards more acid conditions. In the early stages of the corrosion process a powdery white deposit forms which becomes moist and develops into flakes which can easily be removed. These flakes are largely calcium sulphate and the pH of a water extract is between 6 and 7. The surface becomes increasingly moist and finally assumes a putty-like consistency. Large masses can then be broken away and the particles of coarse aggregate seen embedded in the decomposed cement. At this stage free sulphuric acid is present and the pH is frequently less than 2·0. Small amounts of free sulphur can frequently be detected in the corrosion products.

While a certain amount of work has been done on the production of $H_2S$ in sewage, there is very little published work on concrete corrosion. Various writers have suggested explanations, but the experimental evidence in all cases has been slight (Lea and Desch, 1936; Rodt, 1925; Bunker, 1936). None of these explanations, which are purely chemical in nature, satisfactorily explains the production of free sulphuric acid.

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[1] Work carried out by the author as part of a comprehensive investigation into the problem of the corrosion of sewer structures by hydrogen sulphide, which is being carried out by the Research Staff of the Sewerage Branch of the Melbourne and Metropolitan Board of Works.

[2] Senior Chemist and Bacteriologist, Research Laboratories.

The repeated isolation of *Th. concretivorus* (Parker, 1945) in large numbers from the strongly acid corrosion products, and its ability to produce high concentrations of sulphuric acid, suggests that this organism may play an important part in the process.

## EXPERIMENTAL.

*Isolation of* Th. Concretivorus *from Actively Corroding Concrete.*

Samples of corroded concrete were obtained from Brisbane, Sydney, Adelaide, Swan Hill, Kerang and Geelong as well as several samples from the Melbourne sewerage system. Strains of *Th. concretivorus* were recovered from all samples, and where dilutions were made of the corrosion product, cultures could be grown from 1 ml. of 100,000 or 1,000,000 fold dilutions.

*Examination of Conditions in the Corroding Concrete and the Sewer Atmosphere Relative to the Growth Requirements of* Th. Concretivorus.

As it is characteristic of this form of corrosion, that it only occurs on surfaces exposed to the sewer atmosphere, there is an ample supply of $CO_2$ available for the growth of the organism and further as the corrosion products are strongly acid the pH is suitable for growth.

To determine whether the other nutritional requirements are met, samples of corroded concrete were taken and analyzed for moisture, ammonium and nitrate nitrogen, phosphate, sulphate, free sulphur and free acid.

The moisture content was determined by drying 5 gm. of sample at 103° C. to constant weight. The ammonia, nitrate, phosphate, acidity and sulphate were determined on water extracts of the corroded material. The ammonia was determined by direct nesslerization and the nitrate by reduction with aluminium foil in alkaline solution and subsequent nesslerization of the ammonia formed. The sulphate was estimated by the usual gravimetric method with barium chloride, the phosphate by the phosphomolybdate method and acidity by titration and pH measurement. The free sulphur was determined by drying the material and extracting with carbon tetrachloride in a Soxhlet extractor. The extract was oxidized with bromine in carbon tetrachloride and the sulphate formed determined gravimetrically.

Typical analyses of corroded concrete from the Melbourne main outfall sewer are as follows. The water soluble constituents, ammonia, nitrate, $P_2O_5$ and acidity are expressed as a concentration in the moisture present. The sulphate and free sulphur are expressed as a percentage of the total dry solids.

| | |
|---|---|
| Moisture | 40 p.c. |
| Acidity (as $H_2SO_4$) | 1·5 p.c. |
| pH | 1–4 |
| Ammonium N. | 675 p.p.m. |
| Nitrate N. | 12·5 p.p.m. |
| $P_2O_5$. P. | 52·6 p.p.m. |
| $SO_3$ | 36·7 p.c. |
| $CaSO_4$ | 62·4 p.c. |
| S. | ·01 p.c. |

Sewage contains small amounts of ammonium nitrogen and a certain amount is emitted into the sewer atmosphere. The amount present in the sewer atmosphere was determined by aspirating a known volume of gas through an acid absorbent solution and estimating the ammonia fixed, by nesslerization. The concentration present ranged from 0·2 to 0·7 p.p.m. The ammonia in the corrosion products was therefore probably fixed from the atmosphere by the acid moisture present. The phosphate was thought to occur as a constituent of the cement, and this was proved on analysis of two brands of cement available in Victoria. These cements contained 0·05 and 0·13 p.c. $P_2O_5$, respectively.

The corroding concrete is acid and contains available phosphate and nitrogen in sufficient concentrations with some free sulphur, to constitute a suitable medium for growth and the production of acid by *Th. concretivorus*. The finding of free sulphur fits in with the inability of the organism to utilize $H_2S$ and demonstrates the presence of a utilizable source of sulphur.

*Corrosion of Concrete under Pure Culture Conditions.*

Quantities of 200 ml. of the culture medium used in the isolation of *Th. concretivorus* were distributed into two 1 litre Erlenmeyer flasks and autoclaved. A sterile cylindrical block of neat cement (1 inch diameter 1 inch high) was placed aseptically in each flask, so that part only was submerged in the medium. Sterile sulphur was added to each flask and one flask inoculated with a culture of *Th. concretivorus* (strain A). Owing to the solution of lime from the cement it was neces-

sary to readjust the pH of the contents of the flasks to 5·0 for some days before active growth was initiated. Normal growth occurred in the inoculated flask and incubation was continued for 3–4 months. During growth the sulphur fell to the bottom of the flask and was replaced several times. At the end of the incubation period considerable corrosion was evident on the cement block in the inoculated flask. Beneath the surface of the liquid the cement was converted to a white material (largely calcium sulphate) while the remainder of the block maintained its original grey colour. The deposit could be scraped away, showing that the block had been decomposed to a depth of $\frac{1}{16}$ inch. The block in the sterile flask showed no signs of corrosion. Although the corrosion in this experiment could be attributed to the activity of *Th. concretivorus* the conditions of exposure were not identical with those in the sewer. The sulphur source here was elementary sulphur, while in the sewer it is primarily present as $H_2S$.

A further experiment was devised therefore in which two neat cement blocks were exposed to an atmosphere of water vapour, $H_2S$, and ammonia, one being inoculated with *Th. concretivorus* (strain A) and the other left sterile. The method of producing this atmosphere is similar to that described in the next experiment.

Evidence of corrosion was found on the inoculated block after three months' exposure, while none was apparent on the sterile control. An analysis of the water soluble and easily removable corrosion products of the two blocks showed the following contents of sulphate and free sulphur.

| | Sulphate sulphur. | Free sulphur. |
|---|---|---|
| Inoculated block | ·033 gm. | ·063 gm. |
| Sterile block | ·021 gm. | ·011 gm. |

The greater amount of sulphate in the inoculated block was suggestive of the ability of *Th. concretivorus* to bring about corrosion and the production of sulphate in an atmosphere of $H_2S$. A more elaborate experiment was therefore planned to confirm this result and to test the ability of several different strains to corrode concrete.

The test atmosphere was prepared to simulate sewer conditions, i.e. 100 p.c. humidity, 0·5–1·0 p.p.m. of ammonia and a high concentration of $H_2S$. In order to accelerate the corrosion, the $H_2S$ concentration used was about 250 p.p.m. which is higher than that usually found in sewers.

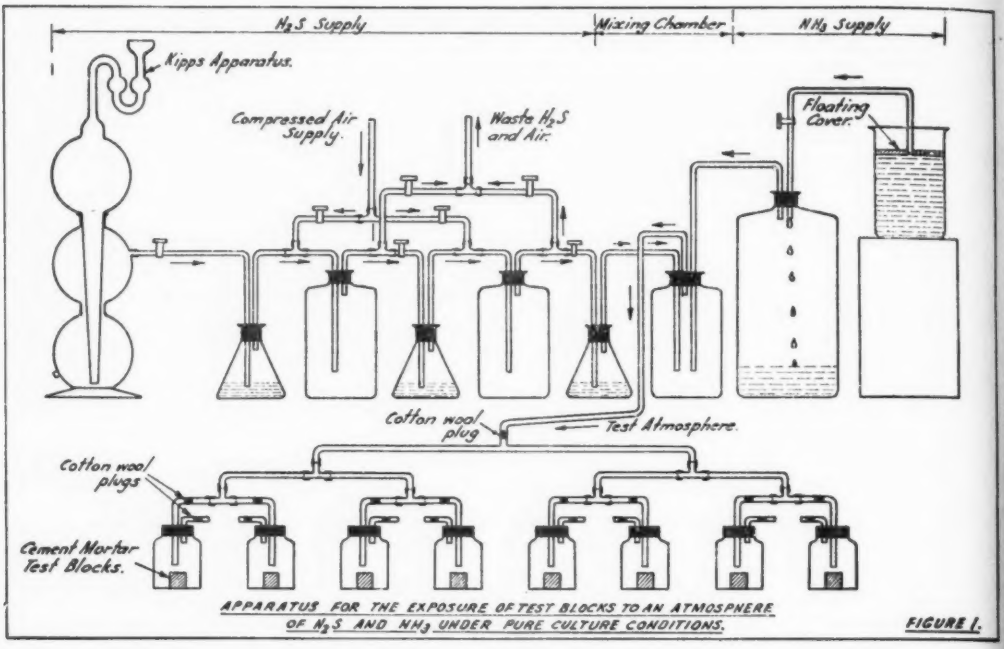
The atmosphere to which the test specimens were exposed was prepared as follows. $H_2S$ was generated in a Kipp's apparatus and this was diluted in two steps with air from the laboratory compressed-air mains to give a concentration of about 350 p.p.m. The dilution was controlled by adjusting the relative bubble rates of the $H_2S$ and air through bubblers. The diluted $H_2S$ was fed into a mixing chamber at the required rate, together with a dilute atmosphere of ammonia, the rate of supply being one-fourth that of the $H_2S$. The ammonia atmosphere was prepared by taking a very weak ammonia solution in tap water and agitating it in a large 5 gallon carboy. The ammonia became distributed between the gas and solution and in this way very dilute ammonia atmospheres of known concentration could be prepared. The ammonia atmosphere was fed into the mixing chamber at the desired rate by displacement with an ammonia solution of the same strength. 100 p.c. humidity was assured by bubbling the $H_2S$ atmosphere through water and the ammonia atmosphere was also in contact with a free water surface. From the mixing chamber the atmosphere (containing about 250 p.p.m. of $H_2S$ and 0·5–1·0 p.p.m. of ammonia) was fed through a distributing arrangement into small bottles containing the specimens to be exposed. This ensured the delivery of the test atmosphere at equal rates to all test specimens. The rate of flow was adjusted so that the air in each bottle was changed once an hour. The apparatus used is shown in Fig. 1.

The specimens exposed to the atmosphere were small blocks (1 inch diameter 1 inch high) of 1: 6 cement-sand mortar. A mortar of this constitution was used because it was expected that visible proof of corrosion would be more readily obtained than with a neat cement. As stated previously it is the cement constituents which are involved in the corrosion process, and therefore a greater volume of disintegration would be expected where these are mixed with a high proportion of sand.

The blocks were distributed, one each into eight small wide-mouthed bottles and each bottle fitted with rubber stopper, inlet, and outlet tube. The inlet and outlet tubes were plugged with cotton wool. The bottles containing the blocks were connected to the distributing arrangement (see Fig. 1) along with which they were sterilized by autoclaving for 30 minutes at 15 lb. pressure, and then connected to the mixing chamber. Of each pair of specimens, one was inoculated with 3 drops of a culture of *Th. concretivorus* while to the other were added 3 drops of the same culture killed by boiling for 5 minutes. In this way the acid present in the culture liquid was added to both blocks. Four different strains (A, B, D and E) were used.

After inoculation the test atmosphere was passed continuously over the specimens for a period of 15 weeks. In order to accelerate establishment of the cultures on the blocks, they were reinoculated weekly for the first six weeks as described above.

By this time obvious signs of corrosion had appeared on all the inoculated blocks.



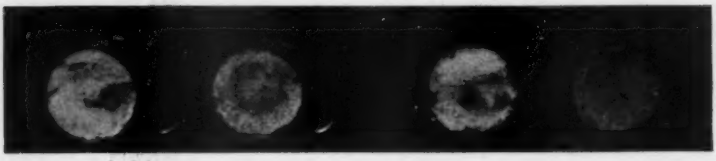

APPARATUS FOR THE EXPOSURE OF TEST BLOCKS TO AN ATMOSPHERE OF $H_2S$ AND $NH_3$ UNDER PURE CULTURE CONDITIONS. FIGURE 1.

The concentrations of $H_2S$ and ammonia in the test atmosphere were estimated from time to time. This was done by sampling the supply of each separately before it entered the mixing vessel. The $H_2S$ was determined by absorption in zinc acetate and colorimetric determination of the absorbed sulphide by the Pomeroy (1936) method. Ammonia was determined by absorption in acid and nesslerization of the absorbed ammonia. The ammonia was found to be between 1–2 p.p.m. while the $H_2S$ content averaged 300 p.p.m. and varied between 150–600 p.p.m.

After six weeks' exposure the inoculated specimens all showed a whitening of the top surface, whereas the sterile control in each case retained the original cement grey colour. This whitening became progressively greater and eventually spread down the sides of the block.

In the early stages of the exposure there was no detectable difference in the $H_2S$ content of the gas leaving the bottles. Towards the end of the 15 weeks approximately 75 p.c. of the $H_2S$ was removed by the inoculated blocks compared with that leaving the sterile controls. At the end of the experiment the inoculated specimens displayed all the signs typical of the normal corrosion process found under sewer conditions. There was no apparent alteration in the controls. This is illustrated by a photograph (Fig. 2) showing the four sets of specimens after exposure. The whitened portions of the inoculated specimens were soft and could easily be scraped away from the unchanged material beneath. The difference in appearance of one pair of samples before and after the corroded material had been removed from the inoculated specimen is illustrated in Figs. 3 and 4.

The corrosion products were acid in reaction and gave a positive test for sulphate. Analyses were made of the inoculated and sterile specimens. Each specimen was first extracted with 50 ml. of distilled water. The pH, the ammonia and the sulphide content of this extract were determined, and the sulphate present estimated by the usual gravimetric method on an aliquot portion. The remainder of the specimen was then completely digested with concentrated HCl and filtered. It was noticed that on the addition of acid, particularly to the sterile blocks, the grey surface immediately became coated with a yellow solid, presumably sulphur. This suggests that polysulphides were formed during exposure of the blocks particularly under sterile conditions. Sulphates in the acid extract were determined after removal of silica and iron. The residue on the filter paper was dried and oxidized with nitric acid, bromine water and bromine in carbon



Inoculated Sterile Inoculated Sterile
Strain A Strain B

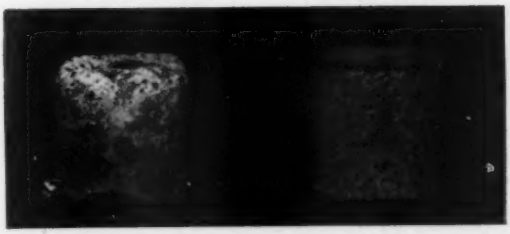
Inoculated Sterile Inoculated Sterile
Strain E Strain D

**Fig. 2.** Cement mortar blocks inoculated in pairs with living and killed cells respectively of four strains of *Th. concretivorus* after 15 weeks' exposure.

Inoculated Sterile
Strain D

**Fig. 3** (left). Blocks (Strain D) before removal of corroded material.

**Fig. 4** (right). Blocks (Strain D) after removal of corroded material.



Inoculated Sterile
Strain D

tetrachloride, the sulphur present being determined as sulphate by the gravimetric method. A specimen block of mortar which had not been exposed to the test atmosphere was also analysed for its sulphate content as an unexposed control. These analyses are tabulated in Table 1.

TABLE 1.

| Analysis.* | Nature of specimen. | Strain A. | | Strain B. | | Strain D. | | Strain E. | |
|---|---|---|---|---|---|---|---|---|---|
| | | Inoc. gm. | Sterile. gm. | Inoc. gm. | Sterile. gm. | Inoc. gm. | Sterile. gm. | Inoc. gm. | Sterile. gm. |
| Sulphate "S" | (Content of exposed block, water + acid extract) | ·403 | ·081 | ·242 | ·086 | ·374 | ·089 | ·189 | ·089 |
| „ „ | (Content of unexposed block, acid extract) | ·047 | ·047 | ·047 | ·047 | ·047 | ·047 | ·047 | ·047 |
| „ „ | (Formed on exposed block) | ·356 | ·034 | ·195 | ·039 | ·327 | ·042 | ·142 | ·042 |
| Free S | (Formed on exposed block) | ·113 | ·099 | ·085 | ·111 | ·053 | ·091 | ND† | ND |
| Sulphide "S" p.p.m. | (Formed on exposed block) | 0·1 | 0·4 | 0·1 | 0·4 | 0·1 | 0·2 | 0·1 | 0·2 |
| Ammonia N p.p.m. | Water extract, exposed block) | 4·0 | 0·5 | 1·0 | Tr. | 2·5 | Tr. | 1·5 | 0·4 |
| pH | (Water extract, exposed block) | 4·0 | 6·8 | 6·0 | 6·7 | 6·2 | 6·6 | 6·0 | 6·6 |

* Sulphate sulphur and free sulphur contents are expressed in grams weight, the sulphide and ammonia contents of the water extracts are as parts per million of the extract.

† ND: Not determined.

The visual evidence of corrosion of the inoculated blocks is confirmed by the analyses. Considerable amounts of sulphate had been formed on the inoculated blocks while the sterile ones showed very little sulphate formation. The blocks inoculated with strains A and D and which showed most visible corrosion were found to have 8 to 10 times the amount of sulphate formed on the sterile controls.

Despite the very small amount of sulphate formed on the sterile controls, there does appear to be a slight conversion of $H_2S$ to sulphate under sterile conditions. Some of this would be due to the sulphate content of the killed inoculum added to these blocks, but this would not amount to more than 10 mg. It is also possible that individual variation in the preparation of the blocks could result in a low sulphate content in the control, compared with the exposed blocks. However, it appears that, while rapid and typical corrosion and sulphate formation can only take place through the activity of *Th. concretivorus*, very small amounts of sulphate can be formed by non-biological means.

Free sulphur is formed on both sterile and inoculated blocks, although the method of estimation was such that it could have been present in any form which would produce the element by reaction with HCl. The behaviour on the addition of acid suggested that some of it was not present as elementary sulphur on the blocks. It appears that this sulphur is present as an intermediate compound formed non-biologically and that the typical corrosion process can only develop where sulphur in this form is converted to sulphate by the activity of *Th. concretivorus*.

Only very slight amounts of soluble sulphide sulphur were present. The ammonia content as expected was higher on the inoculated blocks than on the sterile controls, while the pH of the inoculated blocks was much lower than that of the sterile ones.

## DISCUSSION.

It has been shown that in the presence of pure cultures of *Th. concretivorus*, cement-containing material, such as concrete or cement mortar exposed to an atmosphere of $H_2S$ and ammonia, undergoes rapid corrosion similar to that

observed in practice. Sterile specimens exposed to the same atmospheric conditions do not exhibit these signs of corrosion. The rapid corrosion of concrete structures must be described therefore as a form of bacterial corrosion in which the causative organism is *Th. concretivorus*, the corrosive process being due to the biochemical activities of this organism.

It is difficult to reconcile the fact that the organism in culture medium does not oxidize $H_2S$, with the observation that pure cultures in the presence of cement compounds rapidly absorbed and oxidized $H_2S$ with the formation of sulphate. Apparently under the experimental conditions, free sulphur or poly-sulphides are formed as intermediate products by chemical action between $H_2S$ and the cement compounds, and the organism utilizes these intermediate products for its oxidative activity. However, further chemical examination of the intermediate products is necessary to clarify this point.

As noted above, the surface of concrete exposed to normal atmospheric conditions attains stability with a pH of 8·4, and *Th. concretivorus* could not proliferate under such conditions. This normal condition of concrete must therefore be modified in some way so that the pH falls below 6·5 before the organism can exercise its normal biochemical activity in the presence of $H_2S$. The corrosion process may therefore be divided into two stages, viz. (1) a preliminary stage in which the carbonate-bicarbonate–$CO_2$ equilibrium is destroyed and the pH falls from 8·4 to between 6 and 7; (2) a final stage in which active proliferation and acid formation by *Th. concretivorus* take place.

In the experimental work the preliminary stage was achieved artificially, by inoculation with an acid culture thereby lowering the pH to that required by the organism. Once the necessary pH had been established, proliferation and corrosion took place. The essential function of *Th. concretivorus* has been demonstrated therefore only for the final stage of rapid corrosion.

It is unlikely that *Th. concretivorus* plays any part in the preliminary stage of corrosion, owing to the prevailing pH. It is possible that purely chemical oxidation of sulphur compounds is responsible, but the fact that pH of water extracts of the sterile blocks was only 6·6–6·9 suggests that purely chemical changes are insufficient to achieve the desired acidity. Work now in hand suggests that other species of sulphur bacteria are responsible for this preliminary stage of the process.

From the foregoing discussion of the corrosion process, it becomes possible to explain the behaviour of test specimens exposed to corrosive sewer conditions. Such specimens show little apparent corrosion for 18 months to 2 years, after which a marked acceleration in the rate of corrosion occurs and rapid destruction of the specimen is apparent. The initial lag period apparently corresponds with the first stage of corrosion in which the environment is becoming favourable to *Th. concretivorus*, while the accelerated rate observed after 2 years' exposure results from proliferation of the organism.

## SUMMARY.

*Th. concretivorus* has been isolated in large numbers from specimens of corroded concrete obtained from widely separated localities and under widely different conditions. Conditions existing in corroding concrete are satisfactory for its growth.

Rapid and typical corrosion of cement mortar, exposed to an atmosphere similar to sewer conditions, only occurred in the presence of pure cultures of *Th. concretivorus*.

*Th. concretivorus* did not utilize $H_2S$ directly but did convert into sulphuric acid, free sulphur or some other utilizable form of sulphur, which was probably produced non-biologically.

The process of concrete corrosion under sewer conditions has been discussed and it is concluded that the final rapidly corrosive stage is due to the activity of *Th. concretivorus*. The explanation of the preliminary stage of slow corrosion is not yet clear.

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# THE PRESENCE OF LARGE AMOUNTS OF URIC ACID IN THE INTEGUMENT OF MAMMALS

by ADOLPH BOLLIGER AND MARGARET H. HARDY[1]

(From the Gordon Craig Research Laboratory, Department of Surgery, University of Sydney, and the F. D. McMaster Animal Health Laboratory, Council for Scientific and Industrial Research).

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In preliminary experiments (Bolliger and Hardy, 1944) it was found that considerable amounts of uric acid occur in the integument of a few mammals and particularly in those which only possess very small amounts of this compound in the blood stream. However, previous workers already seemed to be under the impression that the integument may contain comparatively large amounts of uric acid, but their findings were not taken seriously and discredited on technical grounds (Rothman, 1929). Therefore it seemed to be necessary to prove beyond doubt that uric acid as such occurs in the integument and in approximately the amounts indicated by colorimetric determinations. For this reason an investigation on the isolation and definite chemical identification of uric acid obtained from the integument in general as well as from its components such as hairs and skin, was undertaken in three mammals, a marsupial (Australian phalanger, *Trichosurus vulpecula*, commonly called an "opossum"), an ungulate (sheep) and a rodent (rabbit). The extraction and subsequent identification combined with quantitative estimation, were carried out: (a) On the surface of the intact animal, (b) on hairs, (c) on skin after removal of the hairs.

## METHODS AND RESULTS.

Uric acid was determined by Folin's (1934) method. In addition, the new method (Blauch and Koch, 1939) for the determination of uric acid, which is based on the destruction of uric acid by uricase, was also employed.

### *Extraction and Determination of Uric Acid on the Total Surface of the Animal.*

The animal used for this procedure was the rabbit. It was killed by means of ether and was then washed with ether. After the ether had evaporated the animal was placed in a large saucepan containing about 2 to 4 litres of hot water. The water was brought to boil and the animal turned several times in cases where it was not completely submerged. After boiling for about half an hour the water was poured off and filtered. Another 2 to 4 litres of hot water were added to the carcase and brought to boil for about 20 minutes. After pouring off the second lot of water the same procedure was repeated a third time. The three lots of water obtained from the three extractions were pooled and examined for total uric acid content by the colorimetric method of Folin (1934). The volume of the extracts was then reduced to about 200 ml. by evaporation. At this stage the concentrated fluid contained some precipitates which were removed by filtering or centrifuging. After this the volume was further reduced to about 30 ml. and while hot was acidified with 10 p.c. hydrochloric acid. A precipitate formed which, after standing overnight in the refrigerator, was removed by filtration. After washing the precipitate with water, alcohol and ether, the crude yield was determined. The precipitate was then redissolved in a few ml. of approximately 5 p.c. cold piperazine solution. This was accomplished by grinding the crude precipitate with the solvent several times. After filtering off the insoluble material and acidifying the filtrate with 5N hydrochloric acid, a fairly pure precipitate was obtained. For analysis, however, the crystals, which were still stained brown, were redissolved in a small amount of hot 3 p.c. piperazine solution containing some charcoal. After filtration and reprecipitation with hydrochloric acid this procedure was repeated till a practically white product was obtained. These crystals had no melting point but decomposed at about

[1] Walter and Eliza Hall Fellow in Economic Biology, University of Queensland.

400° C. They gave a definite murexide test and were destroyed by the action of the enzyme uricase. Consequently the product was considered to be uric acid, which was further supported by the marked solubility of its piperazine salt, its sparing solubility in cold water and practical insolubility in organic solvents and by elementary analysis.

$C_5H_4O_3N_4$ Cal.: C, 35·70; H, 2·39; N, 33·3,
Found: C, 35·75; N, 2·47; N, 32·8.

Findings obtained from a rabbit of 2·5 kg. body weight illustrate a typical result. After being killed with an overdose of ether, the surface of the animal was extracted as described with a total of about 9 litres of hot water and the total filtered extract was found to contain 320 mg. of uric acid by colorimetric determination. After evaporation and acidification the crude yield of the precipitate obtained was 420 mg.. After redissolving in cold piperazine and filtration from undissolved material 290 mg. of fairly pure, though still brown-stained uric acid, were obtained.

*The Extraction and Determination of Uric Acid in the Hair.*

For this purpose the hairs were clipped off fairly close to the skin and after weighing and extracting with ether for several hours in the Soxhlet apparatus they were dried overnight at approximately 70° C. After reweighing, the hairs were boiled 4 or 5 times with approximately 40 parts of water for about 20 to 30 minutes each time. Further extractions were not found to be necessary since they contained only a fraction of a milligram of uric acid as determined by the colorimetric method. In some instances the extraction with water was executed in the Soxhlet apparatus but it took about six hours of continuous extraction to obtain removal of most of the uric acid present in the hair.

After determining colorimetrically the uric acid content of the extract, this compound was isolated in crystalline form by concentrating the volume down to about 10 to 20 ml. After acidifying with concentrated hydrochloric acid and after letting the solution stand over night in the refrigerator the precipitate was isolated by filtration or centrifuging. After purifying the precipitate once as described in the previous Section, the uric acid thus obtained from rabbit hair exhibited a high degree of purity. For analytical purposes it was further purified by redissolving in piperazine solution and by subsequent reprecipitation.

The results obtained on three specimens of rabbit hair by the colorimetric and gravimetric methods are listed in Table 1.

TABLE 1.

*Uric acid content of rabbit hair.*

| | Colorimetric method. Uric acid mg. p.c. | Gravimetric method. Uric acid mg. p.c. |
|---|---|---|
| White rabbit | 440 | 370 |
| Grey rabbit | 540 | 480 |
| Grey rabbit | 530 | 470 |

The hairs from the rabbit furnished a remarkably pale coloured extract which lent itself to colorimetric determination. In the case of sheep's wool, brown coloured extracts forming voluminous precipitates in the process of concentration, were obtained. These precipitates were removed by filtration and it was then possible to execute a colorimetric determination in spite of a greenish tint present in the unknown. The results obtained from three different determinations on a sample of wool (lamb) were fairly consistent, namely, 98, 108 and 116 mg. p.c. of uric acid. On further concentration of the extract and subsequent acidification a darkly coloured precipitate was obtained. After the usual process of purification of the precipitate by forming a readily-soluble piperazine salt and subsequent acidification crystals of uric acid were obtained which were still almost black. After a further purification of a similar nature approximately 80 mg. p.c. of uric acid were found to be present in several samples of sheep wool by gravimetric determination.

In the case of the opossum a brown yellow extract with a blue fluorescence (Bolliger, 1944) was obtained. On colorimetric determination widely varying values of uric acid were found depending on the situation of the sample of hair examined and on the age of the animal. At present it may be simply pointed out that the dorsal hairs in fully grown males were found to contain about 100 mg. p.c. of uric acid by the colorimetric and the gravimetric method of determination. Further figures will be given on another occasion.

*Determination of Uric Acid in the Skin.*

After clipping the hairs as close as possible to the skin, the skin was dried in air and then after being cut up into small pieces, extracted with ether in the Soxhlet. Then after drying the pieces in an oven at about 70° C. they were weighed and extracted with at least 5 changes of

hot water using about 40 parts of water for each extraction. In other respects the procedure was the same as with hair but as the uric acid content of skin was found to be about 10 to 20 times smaller than that of similar amounts of hair it was necessary to concentrate the extracts considerably in order to obtain satisfactory results with the colorimetric determination. In addition to the colorimetric determination, the uric acid was also precipitated and isolated as such after concentrating the extract down to a volume of a few ml. By colorimetric determination the water extract of the dried skin of a grey rabbit was found to contain 30 mg. p.c., that of a white rabbit 25 mg. p.c. of uric acid. Dried sheep skin was found to contain 12 mg. p.c. of uric acid. Gravimetrically rabbit skin contained about 25 mg. p.c. and sheep skin about 10 mg. p.c. of uric acid.

*Experiments with Uricase.*

The enzyme uricase was prepared according to the method of Blauch and Koch (1939). The preparation obtained by us was only of moderate potency, 1 gm. of uricase oxidizing 200 mg. of uric acid in lithium carbonate solution in 2 hours. On adding 100 mg. of uricase to 2 c.c. of suitably concentrated extracts of hair or skin from sheep or rabbit it was found that about a third to a half of the uric acid as indicated by the colorimetric test on a tungstic acid filtrate, disappeared in the first two hours. However, when keeping the extract containing the uricase at the suitable temperature of 40° to 48° C. for more than two hours it was found that the chromogenic value diminished further and reached practically zero after 35 to 40 hours. Controls kept at the same temperature without the addition of uricase were found to retain their full chromogenic values.

From these experiments it was concluded that most of the chromogenic properties of the extracts of skin and hair of sheep and rabbit with Folin's reagent represent uric acid, since they are almost completely destroyed (converted into the non-chromogenic allantoin) by the specific enzyme uricase.

## DISCUSSION.

The experimental findings recorded indicate that uric acid is present in large amounts in the integument and particularly in or on the hairs of the opossum, sheep and rabbit. The skin, however, contains only about a tenth or less of the uric acid present in or on the hair and necessarily the question arises if the short length of hairs present in and above the clipped skin contribute most of the acid found. With chemical methods this could not be decided.

In the hair of the rabbit uric acid values as high as 500 mg. p.c. are met with and this means an approximately thousandfold increase over the uric acid content of the blood, which according to recent findings (Blauch and Koch, 1939) contains only from 0·0 to 0·7 mg. p.c. The urine also contains only small amounts of uric acid, and from this finding it was concluded that the rabbit as most other mammals, with the exception of man, the chimpanzee and the Dalmatian coach-hound, has a high uricolytic index (96 p.c.) because most of the uric acid formed is converted in the body to allantoin by the enzyme uricase. Consequently, the finding of amounts of uric acid in the integument (e.g. 300 mg. p.c.) many times greater than those present in the rest of the whole of the body (e.g. less than 20 mg. p.c.) is surprising and amongst other implications may have a definite bearing on the understanding of purine metabolism.

It seems probable that uric acid is constantly excreted by the process of shedding of hair and epidermis. This in turn must reduce the amount of uric acid destroyed by the unknown mechanism postulated from the outcome of metabolic experiments in which the estimated excretion of allantoin and uric acid accounted for only about half the uric acid administered to mammals.

Similar conditions are found in the sheep though its wool contains only about 100 mg. p.c. of uric acid; but it possesses a considerably heavier pelage than the rabbit and consequently the total uric acid content for an equal area of skin may well be even higher in the sheep than in the rabbit. On the other hand, the sheep was found to possess only a moderately high uricolytic index, i.e. 80 p.c. (Hunter and Givens, 1914). The uricolytic index of the Australian opossum (*Trichosurus vulpecula*) has not yet been determined, while the Ame-

rican opossum (*Didelphys virginiana*) was reported to have an index of 79. The Australian marsupial (*T. vulpecula*) in common with Eutherian mammals possesses, as shown experimentally, considerable amounts of uric acid in its hairs, and smaller, though significant amounts in the skin.

Amongst the many problems in connection with the observation of large amounts of uric acid in the hairs, arises the question as to how the uric acid is stored. At present it may even be surmised that the uric acid is attached to the keratin molecule in a comparatively loose connection with this structure because it is readily removed by boiling water. Water of about 25° C. only removes less than a tenth of the total uric acid present and this may represent urates which are secreted by the skin glands and only mechanically adhering to the hairs.

Uric acid or uric acid derivatives have been found in large amounts in the integument of lower animals, for example the scales of the fish and snakes, and even the pterines of butterflies may be considered in the same light. Therefore from a developmental point of view it may not seem very strange to find uric acid in the integument of the mammals.

## SUMMARY.

Considerable amounts of uric acid have been found in the integument of the Australian "opossum" (*Trichosurus vulpecula*), sheep and rabbit. The identity of the compound was established by colorimetric, gravimetric and enzymatic methods. Uric acid was found to be present in much larger amounts in the hairs than in the skin.

*Acknowledgment.* We wish to thank Mr. Neil T. Hinks for valuable technical assistance.

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# DILUTED BLOOD PERFUSION OF THE PNEUMONIC LUNG

by E. R. TRETHEWIE

(From the Institute of Medical and Veterinary Science, Adelaide, and the Department of Experimental Medicine, University of Adelaide).

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It has been shown (Trethewie, 1945) that histamine and deaminating enzyme are liberated from the isolated pneumonic lung of the cat perfused with Tyrode. This work has been extended by blood perfusion of the organ. Since it was necessary to use blood for perfusion from the cat from which the lung was isolated, the blood was diluted in Tyrode solution which ensured a sufficient supply and was also of value for another reason which appears in the discussion.

## METHODS.

*Diluted Blood Perfusion.*

Under nembutal anaesthesia (80–85 mg. per kg., intraperitoneally), 50 c.c. of blood were removed from the aorta and run into a heparinized cylinder (0·7 mg. heparin per c.c. blood). This was then diluted in 9 times its volume of Tyrode. A tracheal cannula was introduced and the right lung removed. The left lung was then perfused via the pulmonary artery with Tyrode, and isolated. Portion of the diluted blood was removed immediately after collection and centrifuged at 4,000 r.p.m. for 30 minutes (control). A portion of the diluted plasma was immediately boiled. The diluted blood was perfused through the lung after warming to 37° C. through a coil. Sedimentation was guarded against by agitating the apparatus. The fluid obtained from the lung was collected for 30 minutes, 30 minutes, and then hourly for 3 more hours. Each sample was immediately centrifuged after collection. Further control samples were taken at 2 hours from the blood flask (experiments 1 to 5, Table 1) and a further control taken from the rubber tubing proximal to the lung (experiment 5, column 4, brackets, Table 1). Similar samples were taken at 4 hours. These samples were also centrifuged. In experiments 6 to 10 perfusion was for 1 hour only, and control samples were taken from the rubber tubing at the beginning and at the end of the hour ($C_1$, $C_2$, Table 1). In some, leucocyte counts and platelet counts were made on the diluted blood. The lung was ventilated at 12 cycles per minute.

*Pneumonic lung.*

The right lung and the perfused lung were treated as described previously (Trethewie, 1945).

*Deaminating Enzyme.*

10γ adenosine per c.c. were added to the diluted plasma, a portion boiled and the remainder incubated for 30 minutes at 37° C. and then boiled. Assays were made on the guinea-pig auricle (Drury, Lutwak-Mann and Solandt, 1938).

*Isolated Guinea-pig Jejunum.*

Boiled samples were tested on the jejunum of the guinea-pig suspended in a bath of Tyrode. Histamine was estimated as histamine acid phosphate. Samples were left in contact with the gut for 12 seconds. A substance like the slow-reacting substance of Feldberg and Kellaway (1938) was also estimated on the jejunum.

## EXPERIMENTAL.

### Liberation of Histamine.

A. *Response of the Isolated Jejunum of the Guinea-pig to "Diluted Plasma".*

The diluted blood was centrifuged on collection and portion of the diluted plasma obtained boiled immediately. The addition of samples of this boiled fluid to the bath sometimes produced

a response of the jejunum within 1½ seconds of injection. Usually, no immediate response was obtained. When such a response did occur, it was rarely due to histamine. This was shown by the fact that while one gut might respond with a contraction equivalent to that produced by a certain amount of histamine, the same fluid when tested on a gut from another guinea-pig showed a delayed response with a latency of some seconds and a smaller contraction. Occasionally, no response was obtained with the jejunum of another guinea-pig. This is shown in Fig. 1. At 1 the response is shown to 0·5 c.c. of control fluid obtained from a cat weighing 3·2 kg., the diluted blood being centrifuged immediately after dilution. It is seen that this shows an activity greater than 0·1γ histamine. At 2 the response given by a gut obtained from another guinea-pig was insignificant. Heparine in a dose equal to that contained in diluted plasma has a similar effect. The activity of the initial sample cannot be due to platelets because they were centrifuged out. Later samples may, however, have some contribution from the platelets, though platelet counts on this fluid showed little change after 1 hour.

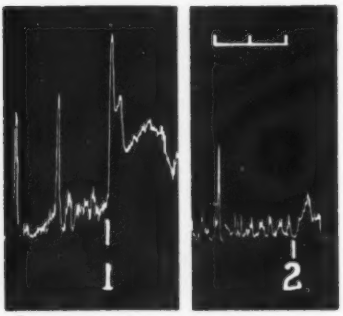


**Fig. 1.** Responses of the jejuna from two guinea-pigs to diluted plasma. Time in minutes. The unlettered contractions are to 0·1γ histamine. Details in text.

In one experiment out of ten the diluted heparinized plasma obtained immediately contained estimable histamine and this was detected in the plasma from the most severely diseased cat. The content of histamine was 1 in 80 millions. After two hours the amount increased to 1 in 10 millions, and after 4 hours was 1 in 12 millions. In another experiment the histamine content after 2 hours was 1 in 50 millions, and after 4 hours 1 in 17 millions (see Table 1).

TABLE 1.

*Output in histamine.*

| Expt. | Lung. | γ Histamine in perfusion fluid. 0–30 mins. | 30–60 mins. | 2 hr. | 3 hr. | 4 hr. | Histamine content diluted plasma (control). 0 hr. | 2 hr. | 4 hr. |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Almost normal | —— | —— | —— | 0·6 | 0·5 | —— | —— | —— |
| 2 | Slightly diseased | —— | 0·6 | 2 | 2·5 | 2·7 | —— | —— | —— |
| 3 | Moderately diseased | 2·4 | 2·4 | 5·3 | 4·6 | 0·5 | 0·017γ/c.c. | 0·1 | 0·08 |
| 4 | Slightly diseased | 1·3 | 0·6 | 1·5 | 78·8 | 4·3 | —— | 0·02 | 0·06 |
| 5 | Slightly diseased | 0·8 | 1 | 1·5 | 1·3 | 2 | —— | —— | —— |
| | | | | | | | (—— | 0·005 | ——) |

| Expt. | Lung. | γ Histamine in perfusion fluid. 0–1 hr. | Leucocyte counts. Number per c.mm. Perfusion sample. $A_1$. | $A_2$. | | $C_1$. | $C_2$. | Boiled diluted blood. |
|---|---|---|---|---|---|---|---|---|
| 6 | Normal | —— | | | | —— | —— | —— |
| 7 | Moderately diseased | 5·4 | | | | —— | 0·0125γ/c.c. | 6 |
| 8 | Slightly diseased | 0·88 | 510 | 580 | 420 | —— | —— | —— |
| 9 | Slightly diseased | 0·9 | 260 | 290 | 400 | —— | —— | —— |
| 10 | Slightly diseased | 1 | 960 | 230 | 310 | —— | —— | —— |

B. *Response of the Isolated Jejunum of the Guinea-pig to Blood Perfused through the Pneumonic Lung.*

(1) *Control.* Blood was obtained from a cat and diluted in Tyrode as described under Methods. Blood was passed through the warming bath exactly as was done in lung perfusions and the fluid was collected through the pulmonary cannula which lay free over the glass plate.

A sample was taken immediately the diluted blood was made up and continuous samples collected from the pulmonary cannula from 0–2 and 2–4 hours. The original sample obtained immediately contained no histamine activity after centrifugation. The first sample obtained 0–2 hours also had no histamine activity on the isolated jejunum of the guinea-pig. The sample obtained 2–4 hours, centrifuged, contained histamine activity 1 in 80 millions.

(2) *Perfusion of the pneumonic lung.* In 5 cases the lungs were perfused for 4 hours and in 5 for 1 hour.

The findings in a case of a moderately healthy lung were as follows. During the first 30 minutes of perfusion no histamine was detected in the centrifuged sample. In the second 30 minutes it contained histamine 1 in 28 millions, and in succeeding hours 1 in 20, 1 in 15 and 1 in 14 millions, giving a total output of 7·8γ histamine. No histamine was estimable in control fluid removed from the flask at the beginning and at 2 and 4 hours. The lung contained 16γ histamine per gm. after perfusion.

These findings are shown in Fig. 2. At $C_1$ is shown the response to centrifuged diluted plasma (1 c.c.) obtained immediately after blood dilution, which had a latency of 4 seconds. A slow reacting response (S.R.S.) is seen (Kellaway and Trethewie, 1939). At $C_2$ a slightly increased similar response with latency 4 sec. is evident from the diluted plasma removed from the reservoir at 2 hours (1 c.c.). At $C_3$ a similar response to $C_1$ is shown by diluted plasma (1 c.c.) obtained after 4 hours. At 1 the response to the diluted plasma (1 c.c.) obtained from the lung during the first 30 minutes' perfusion showed a response with latency 16 seconds. Relaxation was slow and the response suggests that this is not produced by the same material as that found in the control blood. At 2, the response to 1 c.c. of diluted plasma from perfused lung (30–60 minutes) was immediate owing to the presence of histamine. At the arrow the delayed response of the S.R.S. is seen. Similar responses were obtained at 3, 4 and 5 to 1 c.c. of diluted plasma obtained from the second to the fourth hour of perfusion inclusive. Histologically, the right lung showed an occasional lymphoid nodule, emphysema and collections of inflammatory cells between the alveoli. In the case of a more healthy lung, no histamine appeared until the third hour of perfusion and the fourth hour sample only contained histamine 1 in 40 millions. Control fluid contained no histamine. In the one experiment (experiment 4) where sufficient histamine was estimated to enable assay also on the blood pressure of the cat, the finding was comparable with that of the gut. Potassium in the concentration found in the perfusion fluid was without effect on the gut.

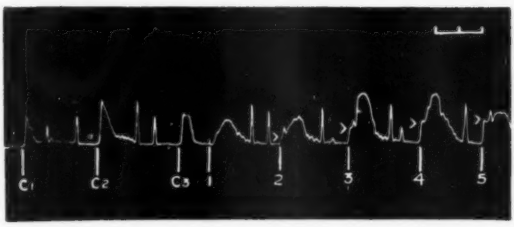


**Fig. 2.** Responses of the jejunum of the guinea-pig to perfusion fluid from lung. Time in minutes. The unlettered contractions are to 0·1γ histamine. Details in text.

Some experiments were further controlled by removing diluted blood from tubing immediately proximal to the lung. Samples were taken at the beginning of, and after 2–4 hours of perfusion, or in the 1-hour experiments just after the beginning and before the end of the experiment. The findings were similar to those of the other type of control sample (Table 1).

### Origin of the Histamine.

It was necessary to show whether the histamine came from the blood or from the lung, since the leucocytes contain histamine. In three 1-hour experiments, a count was made of these cells (Table 1) in the perfused fluid and from the tubing proximal to the lung soon after the commencement $A_1$, and nearing the end $A_2$, of perfusion. It will be seen that in experiment 8, there was a drop of 125 leucocytes per c.mm. in the fluid issuing from the lung. This is insufficient to account for a concentration of histamine 1 in 50 millions as occurred. Also the boiled diluted fluid did not contain any estimable histamine. In experiment 9 there was a rise in the leucocyte count, and in experiment 10 a fall (Table 1). Boiled diluted blood was usually without effect on the isolated jejunum of the guinea-pig in doses of 0·5 and 1 c.c.

### Liberation of Deaminating Enzyme.

A. *Activity of the Diluted Plasma.*

When adenosine was added to diluted plasma obtained from cats, inactivation occurred following incubation. In five experiments, the activity after incubation was 10γ, 8γ, 5γ, 7·5γ and 5γ per c.c. The activity after regular agitation for 2 hours before centrifuging and incubation was 9γ, 8γ, 6γ, 10γ and 6γ respectively, and after 4 hours was 10γ, 7γ, 1γ, 6γ and 7γ respectively. In the third experiment considerable inactivation occurred, especially after standing. This experiment was performed on a cat that was moderately diseased.

B. *Activity of Perfusion Fluid.*

Samples of perfusion fluid were pooled in the proportion in which they were collected, into two lots, namely 0–2 and 2–4 hours of perfusion. The activity of the plasma obtained from the lung was greater than that of the control blood. The activity of the first 2 hours' sample in the first experiment corresponding to the above was 4γ per c.c. after incubation, and the sample 2–4 hours had no cardio-depressant activity after incubation. The next experiment gave 2·5γ and 1γ per c.c. activity respectively after incubation. The third experiment (moderately diseased) gave fluids which completely inactivated following incubation. Similar findings occurred with the fourth experiment.

The numbers of these experiments correspond with the numbers assigned in Table 1. The fifth experiment in A. above does not appear in the Table.

## DISCUSSION.

Since the plasma which was tested after being perfused through the lung had been diluted in Tyrode, substances might pass from the lung to the vascular fluid owing to the presence of a pressure gradient while presumably equilibrium had been present previously between it and the whole plasma. The findings in this paper correspond with those obtained where the lung was perfused with Tyrode (Trethewie, 1945), namely, that histamine and deaminating enzyme are liberated from the pneumonic lung. Difficulties appeared because of the activity of the diluted plasma. The response of the guinea-pig jejunum to the plasma is moderately rapidly acting (Fig. 2); the substance producing a delayed response in the gut which is liberated from the lung produces a slower contraction of the gut with a slower relaxation. It seems likely that these substances are not identical. One would expect this slower acting substance to be present in the plasma before perfusion since it was obtained from the same cat, but it is to be remembered that the blood was diluted in 9 times its volume of Tyrode.

No attempt has been made to indicate the pathway of liberation of the deaminating enzyme and histamine since this was done in the earlier paper.

It is evident that the findings with blood perfusion of the lung correspond to the findings of the Tyrode perfusion and therefore are confirmatory.

## SUMMARY.

Diluted plasma obtained from cats, when tested on the guinea-pig jejunum, shows no estimable histamine.

When the normal lung of a cat is perfused with diluted blood, practically no histamine appears in the perfused fluid.

When the pneumonic lung of a cat is perfused with diluted blood, histamine is liberated into the perfused fluid.

There was an increase in enzyme activity of the fluid which had been passed through the pneumonic lung.

A slow reacting muscle-stimulating substance was liberated from the pneumonic lung.

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# SPONTANEOUS INFECTION OF LABORATORY MICE WITH A PSITTACOSIS-LIKE ORGANISM

by P. DE BURGH AND A. V. JACKSON (MAJORS) AND S. E. WILLIAMS (CAPTAIN) AUSTRALIAN ARMY MEDICAL CORPS

(From an Australian General Hospital and the Walter & Eliza Hall Institute, Melbourne).

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Whilst doing "blind" mouse-lung intranasal passages Nigg (1942) observed a spontaneous infection of her mice by an organism resembling psittacosis in its pathogenicity, morphology and staining characters. Cross immunity and complement fixation tests placed it in the psittacosis-lymphogranuloma venereum-meningopneumonitis group. It differed, however, from typical psittacosis in that it was only pathogenic for mice when inoculated intranasally and was susceptible to the action of sulphonamides (Nigg and Eaton, 1944; Hamre and Rake, 1944).

The object of the present paper is to report the chance discovery of an organism which is, like Nigg's virus, a natural inhabitant of the respiratory tract of mice, but which more closely resembles psittacosis in that it is pathogenic for mice intracerebrally and intraperitoneally as well as intranasally and is not susceptible to sulphonamides.

## SOURCE OF THE VIRUS.

During the investigation of a small human outbreak of "pyrexia of unknown origin" Major P. DeBurgh, pathologist of an Australian General Hospital in Northern Queensland, inoculated blood from one of the patients intraperitoneally into a mouse. Three weeks later the mouse seemed sick and was killed. It had an enlarged spleen and small lesions in the lungs. Spleen and lung from this mouse were pooled and inoculated intraperitoneally into other mice. Spleen and lung intraperitoneal passages were repeated for five passages. All mice used in these passages were subjected to irradiation by X-rays. As, moreover, the small stock of mice at this field hospital was unavoidably kept on a poorly balanced diet which consisted, at times, of only bread and water, it may be assumed that the resistance of these mice was below normal.

Two mice from the fifth passage were sent by air to the Hall Institute, Melbourne. These two mice, when received, had swollen abdomens and appeared feeble and sick. They were killed and found to have a well marked peritoneal exudate with ascites. Smears of the exudate stained by Castaneda's method showed tiny blue staining granules indistinguishable from the L.C.L. bodies typical of the psittacosis group.

On this evidence it seemed possible that the undiagnosed human fevers were actually due to psittacosis virus infection.

However, there was no history of contact with psittacine or other birds. On clinical grounds the diagnosis seemed very unlikely and sera from the patients showed no fixation of complement with psittacosis-lymphogranuloma venereum antigen. Our attention was, therefore, directed to the likelihood that we were dealing with a spontaneous infection in the mice. Accordingly, Capt. S. E. Williams, who had by now replaced Major P. DeBurgh, examined the stock mice in his Queensland laboratory. Lungs from uninoculated mice from the same stock from which came the mice used in the original passages, were inoculated intranasally into other mice. At post-mortem these mice showed irregular pulmonary consolidation associated with large numbers of L.C.L. bodies in smears. It was evident therefore that the stock mice from the Queensland laboratory were infected with this psittacosis-like virus.

Further study of the virus was made at the Walter and Eliza Hall Institute, using material derived from the two fifth passage mice described above. Parallel experiments were performed with a stock psittacosis strain which had been isolated in this laboratory from the spleen of a Gouldian finch. The methods used for the animal and egg experiments were standard for this laboratory. The results of these Melbourne studies are given herewith.

## CHARACTERISTICS OF THE VIRUS.

*In the Mouse.*

All mice used in these experiments were from our Melbourne laboratory stock. Mice from this stock have been used in the past for many experiments involving intranasal inoculations and mouse lung passages and spontaneous infections with a psittacosis-like virus have never been encountered.

*Intraperitoneal.* Peritoneal fluid from one of the fifth passage mice sent to Melbourne was diluted 1 in 10 in 10 p.c. horse serum saline and inoculated intraperitoneally in 0·25 c.c. amounts into four mice. Three died on the 8th, 8th and 9th days respectively. One survived till the 12th day when it was sacrificed. All four mice showed a sticky exudate on spleen and liver and a variable amount of exudate elsewhere on the peritoneum. Smears of the exudate showed L.C.L. bodies.

In later experiments using undiluted egg passage material (allantoic fluid), intraperitoneal inoculation was always fatal in five to seven days.

*Intracerebral.* Six mice were inoculated intracerebrally with 0·03 c.c. of a 10 p.c. suspension of the lung of a mouse which had died three days after intranasal inoculation of the virus. Five of the six mice died on the 4th, 5th, 5th, 7th and 7th days respectively. One survived till the 11th day when it was sacrificed. Meningeal smears showed L.C.L. bodies.

In later experiments using high titre allantoic fluid intracerebral inoculation was regularly fatal in 3 to 5 days.

*Intranasal.* Four mice were inoculated intranasally with 0·05 c.c. of a 10 p.c. suspension of the brain of a mouse which had died four days after intracerebral inoculation. The four mice died on the 3rd, 4th, 4th and 4th days respectively. The lungs showed the irregular grey gelatinous type of consolidation seen in psittacosis infections. The lungs of the mice inoculated with higher dilutions showed small well-defined easily countable lesions resembling those described by Rudd and Burnet (1941). Smears of the lungs showed many L.C.L. bodies.

*In the Guinea-pig.*

*Intraperitoneal.* Two guinea-pigs were inoculated intraperitoneally with 1·0 c.c. of 2nd egg passage allantoic fluid. One pig had a temperature from 104° F. to 105·2° F. for three days after inoculation but otherwise appeared and remained well. The other pig was afebrile and well throughout.

*Intradermal.* Intradermal inoculation of guinea-pigs with 0·1 c.c. of allantoic fluid containing the virus, produced a reddened indurated lesion similar to that caused by psittacosis infection (Bedson and Western, 1930).

*In the Developing Chick Embryo.*

When inoculated by the appropriate route into developing hen eggs the virus grew readily on the chorioallantois (producing in suitable dilution pocks similar to those described for psittacosis by Burnet and Rountree (1935) in the amniotic cavity, in the yolk sac and after one yolk sac passage, in the allantoic cavity. Williams (1944) has shown that psittacosis virus will grow readily and to a high titre in the allantoic cavity. In view of this, allantoic passage of the mouse virus was continued for 11 passages in an attempt to obtain high titre material for use as antigen for complement fixation. Using the intranasal technique of Rudd and Burnet (1941), mouse lung titrations of allantoic fluid showed only a ten-fold increase between the 2nd and 11th allantoic passages (Table 1).

TABLE 1.

*Mouse lung titres of allantoic fluid virus.*

| Virus dilution. | 2nd allantoic passage virus. | | | 11th allantoic passage virus. |
|---|---|---|---|---|
| 1: $10^5$ | 35 | 21 | 17 | C C C C C C |
| 1: $10^6$ | 3 | 3 | 1 | C 68 46 36 31 31 |

Figures = number of discrete foci in lungs. C = patchy consolidation.

*Resistance to Sulphonamides.*

The members of the psittacosis lymphogranuloma venereum group differ in their susceptibility to the action of sulphonamides. Rake, Jones and Nigg (1942), Thomas and Kobb (1943), Hamre and Rake (1944) and Van den Ende and Lush (1943), have shown that infections with lymphogranuloma venereum and with Nigg's mouse pneumonitis virus are controllable with sulphonamides, while infections with meningo-pneumonitis and psittacosis are not.

An attempt was made to see if our mouse strain could, on these grounds, be distinguished from our Gouldian finch psittacosis strain.

Eight weeks old mice were inoculated intranasally with appropriate dilutions (see Table 1) in horse serum saline of allantoic fluids containing the mouse virus and the gouldian finch psittacosis strain. Half the mice in each group were treated with sulphadiazine and the other half were kept as controls. The sulphadiazine treatment consisted of intraperitoneal inoculations of 7·5 mg. of the drug (made up as an emulsion in 4 p.c. gum acacia and containing 7·5 mg. of sulphadiazine in 0·5 c.c. of emulsion); 2 hours, 24 hours, 72 hours and 96 hours after the intranasal inoculation. The results are shown in Table 2.

TABLE 2.

*Survival times in days of sulphadiazine-treated and untreated mice inoculated intranasally with psittacosis and with mouse virus.*

| Dilutions of allantoic fluid virus. | Inoculation with psittacosis. Sulphadiazine treated. | Inoculation with psittacosis. Untreated controls. | Inoculation with mouse virus. Sulphadiazine treated. | Inoculation with mouse virus. Untreated controls. |
|---|---|---|---|---|
| $10^{-6}$ | 12* 13 13 S† NS NS | 8 10 10 14 S | 9 12 S S NS NS | 7 7 8 11 14 S |
| $10^{-7}$ | 7 8 9 9 10 12 | 6 6 7 7 7 7 | 6 9 9 10 10 S | 6 7 8 9 10 10 |
| $10^{-8}$ | 5 6 6 10 10 11 | | 5 5 5 6 8 9 | |

12* = died 12 days after inoculation. S† = Survived 14 days. NS = Nonspecific death.

It will be seen that the treated mice, in both the psittacosis and the mouse virus groups survived on the average slightly longer than the untreated controls. It is doubtful however if this difference is of any real significance. In any case, it is evident that no distinction can be made between psittacosis and our mouse strain on the basis of differing susceptibility to sulphonamide action.

*Antigenicity.*

Attempts to prepare complement fixing antisera by intravenous inoculation of fowls (Hilleman and Gordon, 1944a) with the mouse virus were unsuccessful and so comparison with psittacosis could not be made on this basis. However, allantoic fluid antigens from the Gouldian finch psittacosis strain and from our mouse strain both showed complement fixation with the same dilution of serum from a patient with lymphogranuloma inguinale.

## DISCUSSION.

The mouse pneumonitis virus of Nigg (1942) and a similar virus discovered by Hilleman and Gordon (1944b) may cause spontaneous pulmonary infections of laboratory mice. However, they can be readily distinguished from psittacosis virus by their lack of pathogenicity for mice by the intracerebral and intraperitoneal routes and also by their susceptibility to sulphonamides, and so, any confusion caused by their chance appearance during mouse studies of psittacosis should be readily resolved.

The importance of the virus described in this paper lies in the fact that its close resemblance to psittacosis virus might easily lead to confusion and error. The conditions under which this virus was recovered serve to emphasize the danger of repeated mouse lung passages; and the danger is increased if one uses mice whose resistance has been lowered, deliberately or unintentionally, by any means.

## SUMMARY.

A virus which could not be distinguished from psittacosis virus. has been found to cause a naturally occurring infection in mice.

*Acknowledgments.* We wish to thank Dr. F. M. Burnet for advice and criticism and Maj.-Gen. S. R. Burston, Director-General of Medical Services, for permission to publish this paper.

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# THE ROLE OF BORON IN PLANT METABOLISM

## 2. AN ACCOUNT OF SOME ATTEMPTS TO ISOLATE BORON-COMPLEXES FROM PLANT TISSUES.

by MALCOLM E. WINFIELD[1] (formerly SMITH).

(From the Division of Plant Nutrition, University of California).

*(Accepted for publication 9th March, 1945.)*

Boron is the first micronutrient whose function has been studied which cannot be classified as a heavy metal. It would be expected to resemble phosphorus rather than the heavy metals in the cell reactions of its compounds, but no specific cell reaction involving boron has yet appeared in the literature. A theoretical development of the biochemistry of boron was therefore attempted, as a basis for subsequent experiment.

Boron exists in the solid phase of soils as a constituent of complexes such as tourmaline, and less frequently of soluble salts such as borax, colemanite, and magnesium borate. The less soluble complexes and the sparingly soluble metaborates (e.g. of barium, silver and lead) in addition to boron associated with clays, probably account for the insoluble and unavailable boron. Whatever the original mineral, the boron that is in aqueous solution in soils will be predominantly in one of a few forms, depending upon the pH. In slightly alkaline soils the most stable ion will be $HB_4O_7^-$ or $B_4O_7^=$. In general, however, it can be assumed that the soil solution in which the plant root operates will have a pH less than 7, so that the most important state will be $H_3BO_3$. From the equilibria

$$H_3BO_3 \rightleftarrows H^+ + H_2BO_3^- \quad (K = 1{\cdot}7 \times 10^{-9})$$

$$2H_2BO_3^- + 2H_3BO_3 \rightleftarrows B_4O_7^= + 5H_2O$$

$$B_4O_7^= + H^+ \rightleftarrows HB_4O_7^=$$

it can be calculated that in a culture solution of pH 5·0 containing 0·5 p.p.m. of boron, practically all of the boron will exist as $H_3BO_3$ and only $85 \times 10^{-6}$ p.p.m. as $H_2BO_3^-$, assuming negligible concentrations of polymers of boric acid (Thygesen, 1938).

After entering the cell, it is inconceivable that boron can exist in appreciable concentration as a simple molecule or ion, because $H_3BO_3$ undergoes complex formation with a number of compounds known to be cell constituents. It is assumed then that boron is present in plant cells as one or more derivatives of boric acid. Among the cell constituents which should be considered as possible complex-formers are: d-fructose, d-galactose, α-d-glucose, α-l-sorbose, d-arabitol, meso-erythritol, glycerol, d-mannitol, persitol, pyrocatechol, pyridoxin, citrate, tartrate, calcium gluconate, salicylic acid, saligenin and the tannins of catechin, catechol, and pyrocatechol. In addition there are many other plant compounds which, although there is as yet no good evidence, would probably undergo complex-formation in an aqueous solution of borate. Examples are d-arabinose, d-mannose, l-rhamnose, d-ribose, dulcitol and other hexitols, an unidentified constituent of vitamin P, some flavones, ascorbic acid, d-galacturonic acid, d-glucuronic acid, d-mannuronic acid, and some polymers of sugars and sugar acids.

In a general way, the reactions between boric acid and organic compounds containing two or more hydroxyl groups (either on adjacent carbon atoms, or on two carbons separated by a third as in certain cyclic compounds) are represented in the following equations:

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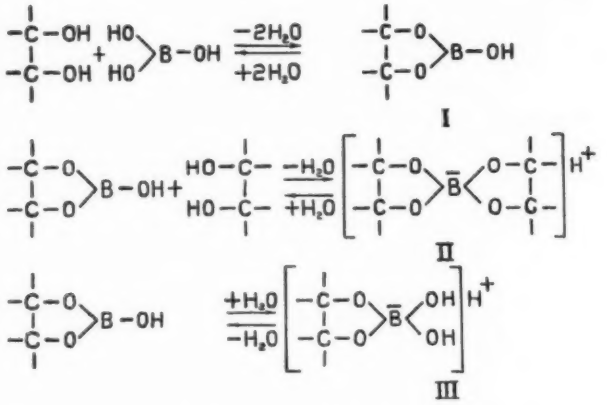
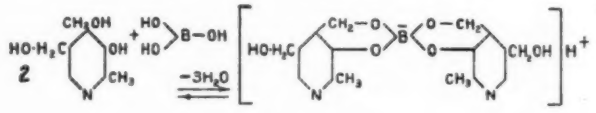
[1] Present address: School of Chemistry, University of Melbourne.



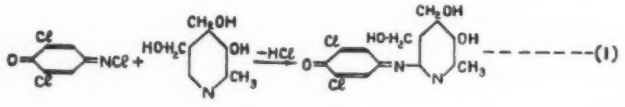
From these equilibria, and the acid strength of II and of III, it is possible to deduce which is the predominant state under various conditions of pH and dilution. It is clear that for the very dilute solutions in plant tissue there will be little complex formation unless the organic compound concerned has a great affinity for boric acid. It is assumed here that the complex to which boron owes its function is the most stable one that can be formed from the hydroxy compounds available in the cell. The stability of boric acid complexes increases with the number of possible resonance structures, and is at a maximum in complexes of type II. Therefore in spite of the opposing dilution effect, which favours types I, III and $H_3BO_3$, it seems likely that the important state in the living cell will prove to be II, more or less dissociated into ions according to the pH. Also implied in the above equilibria is that when plant material is treated with water, during attempted separations of its constituents, the dilution will result in hydrolysis of the borate complex or complexes. Even the pH changes accompanying killing of tissue and expression of sap will influence the equilibria.

Although there is no experimental evidence for a metabolic reaction involving boron, it is possible to give theoretical illustrations of the way in which boron could influence (i) a biological oxidation, (ii) a hydrolysis.

(i) Boric acid and pyridoxin undergo in aqueous solution the reaction,



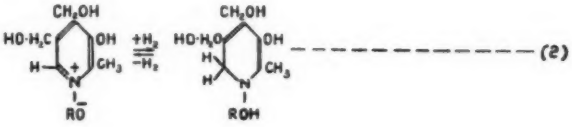
As a result the condensation of pyridoxin and 2, 6–dichloroquinone chloroimide will not proceed in a borate buffer (Scudi, Bastedo and Webb, 1940).



This may be explained by assuming either that the ease of ionization of the hydrogen on the 6 carbon atom is reduced, or that the stability of the imide condensation product is reduced. Either explanation leads to the conclusion that complex formation induces in the pyridine ring a new electronic distribution, which suppresses resonance structures that can be written with a double bond between the nitrogen and the 6 carbon atom. In support of this conclusion is the observation that a solution of pyridoxin alone, at pH 3 or less, exhibits a single absorption maximum which is depressed as the pH is raised to 7·5, two new bands appearing (Scudi, Bastedo and Webb, 1940). The phenomenon is ascribed to structural changes involving the nitrogen

atom and the phenolic hydroxyl group. Borate suppresses the change, and although this may be due entirely to elimination of the phenolic group, it is likely that the borate also deters formation of quaternary nitrogen.[2]

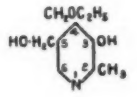
Kuhn and Löw (1939) were unable to confirm experimentally the hypothesis that pyridoxin by analogy to nicotinamide, is part of an enzyme system[3] involved in hydrogen transfer according to equation 2.




(2)

However in view of the above conclusions regarding suppression of reaction (1) it is possible that reaction (2) will proceed in the presence of borate, because the latter will favour the $\diagdown\!\!\diagup\!\!\mathrm{N}\!\!\diagdown\!\!\diagup$ rather than the $\diagdown\!\!\diagup\!\!\overset{+}{\mathrm{N}}\!\!\diagdown\!\!\diagup$ configuration of the nitrogen atom, and it is in the direction left to right that the model reaction would not proceed.

If this picture of the way in which boric acid could catalyze a reversible reduction should prove to be more than a mere illustration, it would be in fair harmony with existing knowledge of the metabolism of boron and pyridoxin. Boron would be necessary to the plant as part of a coenzyme (resembling Coenzyme I with nicotinamide replaced by pyridoxin-boric acid) involved in the synthesis of fat from protein (McHenry and Gavin, 1941). Boron deficiency in higher plants has been shown to be associated with a decrease in fats (Johnston and Dore, 1929; Marsh and Shive, 1941; Lorenz, 1942) and an increase in protein (Lorenz, 1942; see however Wadleigh and Shive, 1939; and Schropp and Arenz, 1940). Pyridoxin is necessary for excised roots of some plants and is highly specific (Robbins, 1942). The substituents on carbon atoms 3 and 4 are important and cannot be replaced by slightly different groups without complete loss of activity. For animals, however, compounds such as




have some activity (Unna, 1940) which may be due to their ready conversion to pyridoxin, or perhaps to a somewhat different rôle of pyridoxin in animals (vitamins such as thiamin are known which are constituents of several different enzyme systems). Whether or not animals need boron (Hove, Elvehjem and Hart, 1939; and an unpublished suggestion that boron is concerned in the lactation of rats) could therefore be independent of its necessity for plants.

(ii) Hydrolysis, and the reverse process of condensation, is another type of reaction to which the biological importance of boron might be attributed. Boric acid could find a rôle analogous to that of phosphate in starch hydrolysis and synthesis (paper number 3 in this series) but it is doubtful whether there is a compound in the cell whose reaction with boric acid involves an energy change of sufficient magnitude to facilitate a biological reaction of this type. It has been shown above that boric acid can act as a negative catalyst in a condensation (reaction 1). There is a number of condensations for which boric acid is a positive catalyst. *In vitro* they are customarily conducted in non-aqueous solution in order to influence the equilibrium in favour of water removal. Since condensations are known to occur in the cell, it must be concluded that under the influence of enzymes these reactions proceed appreciably *in vivo*, even though they do not *in vitro*, in the presence of water. It is therefore justifiable to consider as an example of the way in which boron could catalyze a biological condensation the synthesis of riboflavin from alloxan and dimethyl-amino-ribamine.

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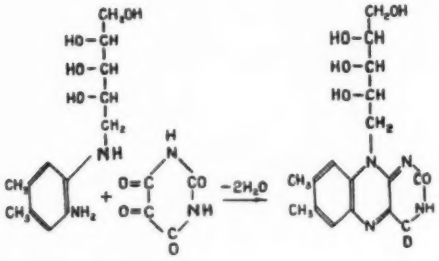
[2] Quaternary nitrogen would be favoured by rising pH, and its appearance would have an influence on the absorption spectrum.

[3] Actually the methyl-iodo-compound of pyridoxin was used as a model, just as in the case of nicotinamide. For the latter RO *in vivo* consists of d-ribose, adenine and phosphate, and it is expected that RO for pyridoxin will prove to be an analogous type of side-chain.



The reaction was conducted in acetic acid solution and was found to be strongly catalyzed by boric acid (Kuhn and Weygand, 1935). These authors suggested that the influence of boric acid is independent of the ribose side-chain[4], and is analogous to the boric acid catalysis of a number of other flavine syntheses (Dimroth and Faust, 1921).

## EXPERIMENTAL.

*Conductivity Changes as an Indication of Complex Formation.*

In order to predict the form in which boron exists in the cell, it is necessary to know the compound which has the greatest affinity for boric acid under biological conditions. Theoretically compounds can be classified approximately with respect to their complex-forming ability by a consideration of the number of possible resonance structures of the complex. No reliable method for comparing the relative affinities experimentally has been developed. If the complexes have sufficiently characteristic absorption spectra, spectrographic examination would provide a convenient method. Alternatively pyridoxin might be employed as a standard for such a comparison. As the pH of the solution increases from 3 to 7·5 the absorption spectrum of pyridoxin develops two new bands, which are suppressed by addition of boric acid (Scudi, Bastedo and Webb, 1940). Subsequent addition of the compound to be tested should result in reappearance of the two bands if it has a more stable boric acid complex than has pyridoxine.

TABLE 1.

| Substance. | $\Delta'$ in dilute solution at 22° C. (mho × $10^6$). | Substance. | $\Delta$ in 0·5 m. solution at 25° C. (mho × $10^4$). |
|---|---|---|---|
| fructose | − 3·4 | glycerol | 9 |
| sucrose | − 0·2 | rhamnose | 23–40 |
| starch | − 4·2 | galactose | 55–49 |
| pyrocatechol | + 0·2 | glucose | 70–23 |
| d-glucose | − 1·3 | erythritol | 64 |
| d-galactose | − 1·7 | pentaerythritol | 72 |
| mannitol | − 1·0 | arabinose | 101–93 |
| d-ribose | + 0·2 | xylose | 205–185 |
| lactose | + 3·9 | mannose | 289–319 |
| gum arabic | − 2·8 | pyrocatechol | 516 |
| pectin | + 0·8 | mannitol | 685 |
| glycogen | − 8·0 | dulcitol | 707 |
| pectinic acid | − 1·6 | fructose | 774–778 |
| ascorbic acid | + 10·8 | sorbitol | 794 |
| galacturonic acid | + 19·1 | | |
| pyridoxin hydrochloride | + 182 | | |

[4] Riboflavin itself undergoes complex formation with borate in aqueous solution, as indicated by the change in optical rotation of a solution of riboflavin in 0·04 molar NaOH from $[\alpha]_D^{20}$ − 100° to + 350° on addition of $H_3BO_3$ (Kuhn and Rudy, 1935). Presumably the combination is with hydroxyl groups of the ribose substituent. To this we have been unable to attach any biological significance by experiments *in vitro*, nor have we been able to devise appropriate experiments *in vivo*.

In the absence of spectrographic equipment, an approximate comparison for certain cell constituents was made by determining conductivity changes in very dilute solutions (Table 1). The equivalent weight of some of the compounds was not known, so in each case 7 mg. of the compound to be tested was dissolved in 20 ml. of water, and the conductivity measured. Boric acid was then added to make the boron concentration 20 p.p.m., and the conductivity determined immediately after shaking. The increase in conductivity ($\Delta'$) was obtained by subtracting the calculated conductivity of the mixture (assuming no complex formation) from the experimentally determined value. For comparison some values of $\Delta$ obtained by Boëseken (1921) for 0·5 molar boric acid solutions are also presented in Table 1. It will be noticed that some compounds (e.g. mannitol and fructose) which have a large $\Delta$ in concentrated solution have a zero or negative value in dilute solution. The greatest conductivity change was obtained in the case of pyridoxin hydrochloride.

*Attempted Isolation of Boric Acid Complexes from Plant Material.*

In some plants much of the boron is in the cell wall and in a relatively insoluble form (Smith, 1944) so that it cannot be isolated as the original complex. Much of the boron in the protoplasm is in solution and it should be possible to isolate this in its native form provided hydrolysis can be avoided. Some boric acid complexes were prepared and their properties examined in the hope of finding characteristics which would facilitate their isolation from a complex mixture such as the protoplasm. Instead they were found to be very soluble in water and generally dissociated by precipitating agents. Some however were precipitated unchanged by alcohol. Accordingly an isolation of a citric-boric acid complex from lemons was attempted but with no success.

To obviate decomposition of boric acid complexes by pH and concentration changes incident to killing and extracting tissue in the presence of water, an effort was made to work in non-aqueous media. Seeds of *Vicia faba* stripped of their seed coats were cooled by mixing with granular dry-ice in a beaker cooled in a mixture of acetone and solid carbon dioxide. The mixture was ground in a cooled meat chopper and the product immediately placed under vacuum in a flask set in the acetone-dry ice mixture, when the carbon dioxide rapidly sublimed. The flask was permitted to return slowly to room temperature, and the vacuum maintained for a week before extracting the meal. It was hoped that the final product contained a negligible amount of water, without having suffered appreciable destruction of the less stable cell constituents. Samples of the bean meal were extracted with dry acetone, ethyl alcohol, benzene, chloroform, ethyl ether, and petroleum ether from which the last traces of water had been removed. None of these solvents was found capable of extracting boron.[5]

*Pyridoxin and Boron Deficiency.*

In consequence of the theoretical interest of pyridoxin to the question of boron metabolism it was necessary to determine whether there are any obvious interrelations between pyridoxin and boron in plant growth. Four groups of Hubbard squash seedlings exhibiting boron deficiency symptoms were set up in the greenhouse in boron deficient culture solution (Smith, 1943) with (a) 1 p.p.m. of boron added as $H_3BO_3$, (b) 1 p.p.m. of both boron and pyridoxin, (c) 1 p.p.m. of pyridoxin alone, (d) no addition. After five days no differences could be detected between plants which received pyridoxin alone and those to which no addition of any kind was made. The roots in group (b) were healthier in appearance than those in groups (a), (c) and (d) but the only growth in any treatment was the production of new lateral roots by plants which received boron only. It was thought that failure to produce new roots by plants in group (b) was due to pyridoxin retarding boric acid absorption by combining with it to yield a difficultly absorbed complex.

A further experiment was conducted with Hubbard squash plants grown for eleven days in culture solution containing 1 p.p.m. of boron. They were transferred to boron deficient solutions and again divided into four groups as above, but in this case the concentrations were boron 0·4 p.p.m. and pyridoxin 16 p.p.m. In the course of a week symptoms of boron deficiency developed in plants to which no boron was added, perhaps more severely in those which received pyridoxin. However, there was no satisfactory evidence that the presence of pyridoxin modified the absorption of boron or the onset of boron deficiency symptoms.

*Oxidation of Ascorbic Acid in the Presence of Boric Acid.*

It has been suggested that there is a relationship between ascorbic acid, calcium and pectic substances (Reid, 1943) and it is therefore interesting to consider the possible relation of boron to ascorbic acid. Oxidation of 0·1 p.c. solutions of ascorbic acid by very dilute solutions of methylene blue hydrochloride was found to be inhibited by boron concentrations of 1 to 1,000 p.p.m.

[5] Boric acid itself has about the same solubility in ethyl alcohol as in water and therefore would have been extracted by alcohol if present in the bean meal as uncombined boric acid.

The experiments were conducted in distilled water, employing boric acid solutions, so that the inhibitory effect may have been due to pH changes, although a boric acid solution containing 1 p.p.m. of boron has little influence upon pH.

The influence of boric acid upon the oxidation of $10^{-3}$ molar ascorbic acid solutions by atmospheric oxygen was studied briefly in the presence and absence of copper, in solutions maintained at pH 6·2 by 0·2 molar phosphate buffer. The boron solutions were prepared by adding KOH to $H_3BO_3$ solutions until the pH reached 6·2. Ascorbic acid was determined by titration against 2, 6–dichlorophenol indophenol, before and after aeration of the solutions by a rapid and uniform stream of air bubbles. It will be seen from Table 2 that there was no inhibition by boric acid of the fast oxidation in the presence of copper. The small inhibition of the oxidation of ascorbic acid in the absence of heavy metal may be real but is too weak to be of significance in plant metabolism.

TABLE 2.

| | Copper absent. | | | Copper present. | | |
|---|---|---|---|---|---|---|
| Time of aeration (t) | 0 | 10 | 10 | 0 | 5 | 5 |
| P.p.m. of boron | 0 | 0 | 3·3 | 0 | 0 | 625 |
| Ascorbic acid after time (t) | 100 | 60·2 | 62·0 | 10·8 | 4·8 | 4·8 |

It is desirable to continue studies of this type in buffers other than phosphate (which may compete with boric acid for ascorbic acid) and including the influence of calcium.

*The Relationship between Boron and Borocitrine.*

According to Kuhn (1937) *Penicillium glaucum* produces in a medium containing lactose and asparagine as the sources of carbon, and in the presence of boric acid, a yellow substance with a green fluorescence which he called borocitrine. The mycelium grew fairly well in boric acid concentrations as high as 2·5 p.c. but no conidia were formed in this case. This new yellow dye resembled riboflavin in some respects and although it contained no boron Kuhn believed that it existed originally as a boric acid complex. In contrast to riboflavin borocitrine was stable in alkaline solution, but was rapidly and irreversibly destroyed by acids, even dilute acetic acid. Under a mercury vapour lamp the green fluorescence of borocitrine solutions disappeared immediately on addition of sodium hydrosulphite and reappeared on gentle agitation with air, leading Kuhn to suppose that borocitrine can participate in biological oxidations. Here is an opportunity to learn something of the biological reactions of boric acid, if we can examine the constitution and properties of borocitrine and the influence of boron, calcium and sugars upon its production. However it has not yet been possible to reproduce Kuhn's results. *Penicillium glaucum* grown in a synthetic medium containing $NaNO_3$, $K_2HPO_4$, KCl, $MgSO_4$, lactose, l-asparagine and a trace of iron and zinc failed to yield any borocitrine, irrespective of the amount of boric acid added. Instead a yellow colouring matter with no fluorescence appeared in the media at all boron concentrations.

Comparable results were obtained when the fungus was grown in a standard Dox-Czapek medium to which were added appropriate amounts of zinc, copper and manganese. Growth was excellent at all boron concentrations except 1,000 p.p.m. and higher, when germination was greatly delayed. A very small amount of mycelium slowly developed which was white and lacking conidia, although the latter finally appeared in small numbers after ten weeks. When a good crop of mycelium had developed in a majority of treatments the medium was drained off and made slightly alkaline. Four litres of the yellow liquid were evaporated to small volume on a steam-bath, and extracted with quinoline, yielding a reddish-brown extract with no fluorescence.

*A New Carbohydrate from Boron-Deficient Squash Leaves.*

An unknown compound with extraordinary properties was isolated from boron-deficient Hubbard squash leaves during an attempt to determine the relative activity of phenol oxidases in boron-deficient and normal plants. Extraction of the leaf material by phosphate buffer of pH 8·0, followed by addition to the extract of ethyl alcohol to 40 p.c., yielded a precipitate of brown viscous liquid. The liquid was very soluble in water, from which it was reprecipitated several times by addition of ethyl alcohol. Since it could be dried to a pale brown amorphous powder by successive treatment with alcohol and ether it was apparently a strong aqueous solution, and it is strange that as such it should be precipitated in 40 p.c. alcohol. Preliminary tests indicated that the powder is a carbohydrate. All of the accepted tests for tannins were negative. The substance contains a reducing sugar, is fermentable, and behaves like a sugar on heating. An aqueous solution exhibited no optical activity.

## DISCUSSION.

It is suggested that the important biological reactions in which boron participates are the reactions of boric acid complexes. Further, the principal necessity for boron in higher plants can be attributed to a need for boric acid as a component of an enzyme system, but it is not yet clear whether the enzyme is one concerned with transfer of – H or of $H_2O$. The possibility of the enzyme being one such as pectase concerned with transfer of $-CH_3$ (Smith, 1943) is more remote.

Studies *in vivo* of the function of boron in the cell wall cannot be visualized until biochemical technique is much advanced. Meanwhile the boron and also the calcium in the cell wall (Smith, 1944) can be considered to act as modifiers of the permeability of some component of the wall, possibly protopectin. That this function is minor to the function in the cytoplasm is suggested by the greatly diminished amount of insoluble boron in monocotyledonous plants (Marsh and Shive, 1941). The influence of boron upon certain enzyme systems will be presented in a subsequent paper.

## SUMMARY.

A theoretical basis is developed for further study of the biochemistry of boron. It is suggested that boron enters the plant as boric acid, thereupon combining with one or more of the polyhydroxy compounds which are contained in the cell. A number of such compounds are cited which can form complexes with boric acid under certain conditions, and it is shown by conductivity measurements in dilute solution that one of the most probable complexes in the plant cell is that formed with pyridoxin. The ways in which the chemical properties of boric acid may be utilized by plants in oxidation and in condensation processes are illustrated by reactions involving pyridoxin and riboflavin.

Attempts to isolate a boric acid complex in its native state from plant material were unsuccessful. Addition of pyridoxin to the culture solution has no appreciable influence upon absorption of boron and appearance of boron deficiency symptoms in squash plants grown in culture solutions lacking boron. Inhibition by boric acid of the oxidation of ascorbic acid in the absence of heavy metals is small and probably unimportant. Production of borocitrine by *Penicillum glaucum* in the presence of boric acid (Kuhn, 1937) could not be confirmed. A new carbohydrate has been isolated from boron-deficient squash leaves.

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# THE RELATION BETWEEN URINARY VOLUME AND THE URINARY EXCRETION OF THIAMIN AND RIBOFLAVIN OF A MAN FED A CONSTANT DIET

by CATHERINE E. FRANCIS AND E. C. SLATER

(From the Australian Institute of Anatomy, Canberra, A.C.T.).

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It is generally considered that the amount of thiamin and riboflavin excreted in a day is independent of the urinary volume. Melnick *et al.* (1939) found that a single marked diuresis in one of their subjects, subsisting on a standardized diet and given forcing fluids, was not associated with any increase in urinary thiamin values. Elsom *et al.* (1942) found that the day to day variation in the amount of thiamin excreted by subjects on a constant diet is not correlated with the urinary volume. Little work on the effect of urinary volume on riboflavin excretion has been reported, and in studies on the excretion of riboflavin by subjects on a constant diet (e.g. Williams *et al.* 1943), this factor has apparently not been considered. However, Feder *et al.* (1944) claim that more constant values are obtained if the riboflavin content of urine collected for one hour after overnight fasting is expressed in terms of riboflavin per unit of volume rather than per unit of time.

A number of factors have been found to affect the amount of these vitamins excreted in urine. Harris and Leong (1936) first reported that the urinary excretion of thiamin is dependent upon both the dietary intake of the vitamin and the state of nutrition of the subject. Emmerie (1937) found that riboflavin behaves similarly. Wright and Baker (1939) showed that the 24 hour-urinary excretion of thiamin fluctuates from day to day, reflecting changes in the dietary supply of thiamin. According to Wang and Yudkin (1940) the carbohydrate and fat content of the diet as well as exercise influence the thiamin excretion. Cleland (1943) reported that aspirin affects the excretion of thiamin and Elsom *et al.* (1942) found reduced excretions during an infection. However, if these factors are controlled, a normal subject, when placed on a constant diet, excretes practically a constant amount of thiamin and riboflavin after a day or two (with some daily fluctuations), provided that the diet supplies thiamin and riboflavin sufficient to prevent continued depletion of the tissue reserves.

In general, observations made in this laboratory are in agreement with the view that the urinary excretion of thiamin and riboflavin is independent of the urinary volume and that a constant excretion of thiamin and riboflavin can be obtained after a few days on a constant diet. However, in this paper, we wish to report a case with the following unusual features:

(i) Over a period of 15 days on a constant diet there was a rapid trend downwards in the thiamin and riboflavin excretions, although the intake of the vitamins should have been sufficient to prevent depletion of tissue reserves.

(ii) Superimposed on these trends, there were rather large day to day fluctuations of the thiamin and riboflavin excretions. The general trends of the thiamin and riboflavin excretions and of the urinary volumes corresponded in that they descended more or less uniformly. In addition, the day to day fluctuations of the thiamin and riboflavin excretions were highly correlated with the day to day fluctuations of the urinary volume.

## EXPERIMENTAL.

*The Subject.*

The subject, a male 31 years of age and weighing 75 kg. at the commencement of the experiment, was engaged in a sedentary occupation.

*Experimental Diet.*

The experimental diet eaten each day consisted of 280 c.c. milk, 1 oz. butter, 1 oz. breakfast cereal, 6 oz. white bread, 15 gm. sugar, 2 oz. rhubarb, 3 oz. tomato, 1 egg, 3 oz. peach, 6 oz. corned beef, 8 oz. potato, 4 oz. bean and 190 c.c. of a cordial containing orange juice, lemon juice, tartaric acid and citric acid. The diet was supplemented with 50 mg. of synthetic ascorbic acid. The water intake, which was unrestricted, was not recorded. This diet provided (according to direct analyses) 79 gm. fat, 92 gm. protein, 366 gm. carbohydrate, 2,542 Calories and 0·81 mg. thiamin. The riboflavin content of the diet was not determined experimentally but, by calculation from food tables, it contained 1·51 mg. riboflavin. Expressing the thiamin and riboflavin intake in terms of 1,000 Calories, these figures become: thiamin 0·32 mg. and riboflavin 0·59 mg. per 1,000 Calories.

For a fortnight before the commencement of the experiment, foods especially rich in thiamin and/or riboflavin (viz.: milk, wholemeal bread, porridge, cheese, pork) were either omitted from the diet or eaten only in small quantities. Care was taken to reduce variations in the composition of the foods in the experimental diet as much as possible, e.g. all the potatoes were of the same variety, dug at the same time from the same plot and uniform cooking methods were used.

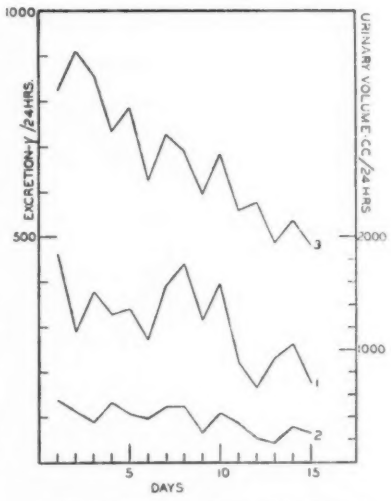


**Fig. 1.** Urinary volume, thiamin and riboflavin excretions. 1. Urinary volume; 2. Thiamin excretion; 3. Riboflavin excretion.

*Collection of Urine and Analysis.*

The urine was collected over 24 hours in amber bottles containing 10 c.c. hydrochloric acid (10 p.c.) and a few c.c. of toluene. Thiamin and riboflavin were determined within a day or two of collection of the sample. Thiamin was determined by the thiochrome method using the technique employed for extracts of food by Slater and Rial (1942). Riboflavin was determined fluorometrically employing the technique of Hodson and Norris (1939) for determining riboflavin in foodstuffs.

The daily excretions of thiamin and riboflavin are plotted together with the urinary volume in Fig. 1.

*General Condition of the Subject.*

The subject showed a considerable loss of weight in the first week, but a slight gain in the second week. The actual weights were: 2nd day 75·1 kg., 9th day 73·2 kg., 16th day 73·5 kg.

The subject developed diarrhoea on the 11th day and this continued until the end of the experiment.

## DISCUSSION.

By inspection of the curves it is apparent that there is a marked correlation between the urinary volume and the excretion both of thiamin and riboflavin.

This correlation may be calculated in two ways. Firstly, the excretion of thiamin and riboflavin may be correlated with the urinary volume over the whole period. This calculation takes into account not only the daily fluctuations of the thiamin and riboflavin excretion and the urinary volume, but also the general trend of the figures over the whole period. Secondly, the day to day change in the thiamin and riboflavin excretion may be correlated with the day to day change

in the urinary volume. If the trends are taken as linear, these correlations are independent of the trends. The various coefficients of correlation are given in Table 1.

TABLE 1.

*Coefficients of correlation.*

| Correlation. | Coefficient of correlation. | Probability that correlation is due to chance. |
|---|---|---|
| Thiamin excretion against urinary volume | + 0·774 | 1 in 500 |
| Riboflavin excretion against urinary volume | + 0·770 | 1 in 500 |
| Δ thiamin excretion against Δ urinary volume | + 0·605 | 1 in 25 |
| Δ riboflavin excretion against Δ urinary volume | + 0·699 | 1 in 100 |

In making these calculations the values for the first two days have been omitted in order to avoid any effect due to "carry-over" of vitamin excretion from the pre-experimental period.

These coefficients show that there is a significant and high degree of positive correlation between the urinary volume and the excretion of thiamin and riboflavin.

The general trend downwards of the urinary excretion of both thiamin and riboflavin suggests that the stores of these vitamins were being depleted rather rapidly. This was not expected at the levels of intake used in this investigation. The intake of thiamin was 0·32 mg./1,000 Calories. According to Melnick (1942), an intake of 0·35 mg./1,000 Calories is sufficient to saturate the tissues. Keys *et al.* (1943) found that the average thiamin excretion was 106γ/24 hrs. in the second week on an intake of 0·33 mg./1,000 Calories and remained about that level for the remaining eight weeks of the experiment; in the tenth week the average output was 92γ/24 hrs. In our experiment the thiamin excretion fell from 123γ/24 hrs. at the beginning of the second week to 63γ/24 hrs. at the beginning of the third week. The intake of riboflavin was about 0·59 mg./1,000 Calories. Williams *et al.* (1943) found only very slight depletion of the tissue stores of subjects fed 0·5 mg. riboflavin per 1,000 Calories.

Moreover, we found no downward trend of the urinary excretion of thiamin or riboflavin in another subject fed at the same time a diet similar to that of the subject considered, but containing less thiamin and riboflavin. In this case, there was no correlation between the excretion of thiamin or riboflavin and the urinary volume.

It appears then, that some abnormality caused an increased requirement for these two vitamins or alternatively, interfered with the normal mechanism of excretion of the vitamins. It may be significant that a large loss of weight (viz., 1·9 kg.) occurred during the first week. Such loss could hardly have been due to tissue catabolism caused by an inadequate calorific intake. The catabolism of such a weight of adipose tissue would provide about 14,700 Calories, i.e. 2,100 Calories per day. This, added to the 2,542 Calories obtained from the diet would mean a total energy supply of about 4,600 Calories—certainly excessive for a sedentary man weighing 75 kg. Possibly the loss of weight was due to an upset in the water metabolism during this period. The subsequent very low urinary volumes in the second week (when there was a slight increase in weight) give some support to this view.

Normally, daily fluctuations of the urinary volume (caused by variations in the water intake, sweat loss, etc.) are not associated with fluctuations in the excretion of thiamin and riboflavin. This case, then, appears to be abnormal. It should be noted, however, that the correlation reported in this paper does not necessarily mean that the excretion of these vitamins is affected *per se* by the blood flow to the

glomeruli. There appears to have been an abnormal requirement for these vitamins. The factor responsible is not clear, but it is possible that this same factor may have been responsible for an abnormal upset in the water metabolism. The volume of urine excreted would, of course, be associated with any upset in the water metabolism. Thus it would be possible, under such circumstances, to find a correlation between the excretion of the vitamins and the urinary volume.

## SUMMARY.

An unusual case is described in which there was a marked correlation between the urinary volume and the excretion both of thiamin and riboflavin of a subject fed a constant diet.

Over a period of 15 days on this diet, which provided, on normal standards, sufficient thiamin and riboflavin to prevent depletion of the tissues, there was a decrease in the thiamin and riboflavin excretions.

It is pointed out that the correlation between the urinary volume and the excretion of thiamin and riboflavin does not necessarily mean that the excretion of these vitamins is affected *per se* by the blood flow to the glomeruli.

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## ADDENDUM.

In this laboratory, Slater and Morell (to be published) have investigated the specificity of eight modifications of the fluorimetric method of determining riboflavin in urine and have found that all but two of these modifications are either not specific for riboflavin or are not quantitative. From their findings, it appears that the method used in the present study gives results which are too high by about 200–400γ/24 hrs. It follows that all the riboflavin excretions shown in Fig. 1 are high. However, as these figures all exceed the true values by about the same amount, the correlation of the riboflavin excretion against the urinary volume would not be affected appreciably.

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# THE EFFICIENCY OF THE HUMAN PROTHROMBIN SYSTEM

by P. FANTL

(From the Baker Institute for Medical Research, Alfred Hospital, Melbourne).

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According to the most generally accepted hypothesis, clotting of blood is the result of a coupled reaction; in the first phase prothrombin is converted into thrombin through the agency of thromboplastin and calcium ions, whilst in the second phase thrombin causes fibrin formation from fibrinogen.

Two opposing views concerning the prothrombin content of human blood have been expressed. Using the technique of Quick (1941) based on the assumption that thromboplastin isolated from rabbit's brain is most active for all mammals, one is led to believe that human plasma contains only about one-fifth of that found in the plasma of rabbit, dog and cat. However, Warner, Brinkhous and Smith (1936), using a different technique find that the prothrombin content of human blood is approximately four-fifths of that of the above mentioned species.

In Quick's technique the two phases take place in the same medium and the plasma concentration is not greatly altered. The other technique requires two independent steps: in a preliminary incubation period in a system freed of fibrinogen, prothrombin is converted into thrombin and the clotting phase is observed after addition of fibrinogen. The quantitative evaluation is carried out by means of serial dilutions. It is the speed of the first phase which indicates the prothrombin concentration since it is assumed that fibrinogen acts merely as an indicator and apart from thrombin is not influenced by other components of the system. However, the conversion of prothrombin to thrombin and the latter's activity is not merely governed by the concentration of prothrombin but by inhibitors (Ferguson and Glatzko, 1941) as well as activators (Quick, 1943) present in the plasma, and for this reason it is better to speak of the prothrombin system. If a comparison of the prothrombin content in two species is to be made in a one-stage procedure it is essential to ensure similar reaction rates for thrombin formation in the different systems. Observing these conditions, comparisons have been carried out on human and rabbit plasma.

## EXPERIMENTAL.

### *The Influence of Homologous and Heterologous Thromboplastin on Plasma Coagulation.*

The thromboplastin from rabbits' brain was prepared immediately and that from human brain four to twelve hours after death of patients who died from diseases or accidents not affecting the brain. The technique of the thromboplastin preparation and the estimation of the plasma clotting time were carried out according to the technique of Quick (1938).

A 6 p.c. brain extract in 0·85 p.c. NaCl and 0·025 M $CaCl_2$ was used. Seven different batches of rabbits' brain and five from human sources were tested. The dried powder was kept in evacuated ampoules in the refrigerator and its age varied from one day to two years. Table 1 includes the results of thirty-three specimens of human plasma derived from venous blood containing 10 p.c. of 0·1 M sodium oxalate.

The blood samples came from hospital patients without clinical signs of prothrombin deficiency but from the results tabulated it is obvious that hypothrombinaemia was detected in several cases. The average clotting time for all specimens was 15·5 seconds when human thromboplastin was employed and 16·9 seconds when rabbit thromboplastin was used. Although human thromboplastin generally gave a shorter clotting time with the homologous brain preparation there are variations in both directions. A calculation of "Students" t (Fisher, 1928) gave a value of 3·30. For $n = 32$ and $P = \cdot 01$, $t = 2 \cdot 74$, so that less than one value in a hundred will exceed 2·74 by chance or in other words, the values obtained indicate that the difference

between the results is significant. In three cases we had the opportunity of taking plasma coagulation time from patients suffering from obstructive jaundice and who had a marked hypoprothrombinaemia. Here again the homologous thromboplastin preparation produced shorter clotting times. It appears, therefore, that the results obtained justify the conclusion that the homologous prothrombin-thromboplastin combination has a greater activity for thrombin formation than the heterologous one.

TABLE 1.

*Coagulation time in seconds of 33 human plasma specimens with rabbit (R.T.) and human (H.T.) thromboplastin.*

| H.T. | R.T. | H.T. | R.T. | H.T. | R.T. |
|---|---|---|---|---|---|
| 15·0 | 18·0 | 14·0 | 17·0 | 14·7 | 14·5 |
| 14·5 | 17·0 | 14·0 | 15·0 | 15·5 | 16·4 |
| 24·0 | 18·0 | 14·0 | 14·0 | 13·6 | 17·1 |
| 22·0 | 19·0 | 14·0 | 14·5 | 16·5 | 19·1 |
| 22·0 | 19·0 | 14·5 | 16·5 | 19·0 | 21·6 |
| 17·0 | 18·5 | 13·5 | 16·0 | 16·1 | 18·5 |
| 17·0 | 17·0 | 12·0 | 14·0 | 12·8 | 16·0 |
| 18·5 | 19·0 | 15·0 | 16·0 | 15·5 | 18·5 |
| 15·0 | 16·0 | 13·5 | 17·5 | 13·8 | 15·8 |
| 15·0 | 17·5 | 13·3 | 14·1 | 15·5 | 14·5 |
| 15·0 | 16·0 | 13·7 | 15·0 | 12·3 | 14·8 |

*The Influence of Temperature on Coagulation.*

The estimation of prothrombin is commonly carried out at 37·5° C. However, it is not known whether the rate of thrombin formation at this temperature is equal in different mammalian plasma.

In the following Table are shown results obtained when clotting was carried out at 37·5° C. and at 22° C. using both the homologous and heterologous combination. The figures represent the ratio of the clotting time at the two temperatures (22°/37°).

TABLE 2.

*Temperature coefficient of coagulation of human plasma (H.P.) using human thromboplastin (H.P. + H.T.) and rabbits' thromboplastin (H.P. + R.T.).*

| H.P. + H.T. | H.P. + R.T. | H.P. + H.T. | H.P. + R.T. |
|---|---|---|---|
| 1·84 | 1·85 | — | 1·78 |
| 1·70 | 1·87 | — | 1·85 |
| 1·55 | 1·95 | 1·60 | 1·65 |
| 1·50 | 1·72 | 1·61 | 1·97 |
| 1·77 | 1·90 | 1·35 | 1·66 |
| 1·65 | 1·80 | 1·71 | 2·00 |
| — | 1·70 | 1·95 | 1·93 |
| — | 1·73 | — | 1·67 |
| — | 1·85 | — | 1·85 |
| — | 1·83 | — | 1·50 |

TABLE 3.

*Temperature coefficient of coagulation of rabbits' plasma using human thromboplastin (R.P. + H.T.) and rabbits' thromboplastin (R.P. + R.T.).*

| R.P. + R.T. | R.T. + H.T. | R.P. + R.T. | R.P. + H.T. |
|---|---|---|---|
| 2·26 | 1·90 | 2·00 | — |
| 2·32 | 2·10 | 2·14 | — |
| 1·95 | 1·82 | 2·15 | 1·93 |
| 2·27 | 1·77 | 2·25 | 1·97 |
| 2·30 | 2·08 | 2·40 | 1·70 |
| 2·38 | 2·07 | 1·90 | — |
| 1·94 | — | 1·86 | — |
| 1·98 | — | | |

The results indicate that human thrombin when produced by homologous thromboplastin shows a lower temperature coefficient whilst rabbits' thrombin produced by a similar process has a higher one; the difference between the temperature coefficients with homologous and heterologous preparations seems to be statistically significant. "Students" t in the case of human plasma (11 specimens)[1] was found to be 4·41; calculated for $n = 10$, $t = 3·169$. In the case of rabbits' plasma (9 specimens)[1] $t = 5·59$; calculated for $n = 8$, $t = 3·355$.

Of further importance in optimal clotting is the calcium concentration. Using both homologous and heterologous thromboplastin combinations a few experiments have been carried out on this point and observations are shown in Table 4.

TABLE 4.

*The influence of varying calcium concentrations on plasma coagulation time.*

| | Human plasma + human brain. $CaCl_2$ concentration. | | | Human plasma + rabbits' brain. $CaCl_2$ concentration. | | |
|---|---|---|---|---|---|---|
| | 0·025 M. | 0·0125 M. | 0·00625 M. | 0·025 M. | 0·0125 M. | 0·00625 M. |
| 1 | 13·3 | 12·9 | — | 14·0 | 13·8 | — |
| 2 | 13·7 | 13·0 | — | 15·0 | 13·6 | — |
| 3 | 14·7 | 12·5 | — | 14·5 | 14·5 | — |
| 4 | 15·5 | 13·0 | — | 16·4 | 14·7 | — |
| 5 | 13·8 | — | 13·0 | 17·5 | — | 14·5 |
| 6 | 15·0 | 14·0 | 13·8 | 16·0 | 15·3 | 15·1 |
| *7 | 17·9 | 16·5 | 17·5 | 20·5 | 18·5 | 17·5 |
| 8 | 14·5 | 13·0 | 13·8 | 16·5 | 14·6 | 14·8 |
| 9 | 13·5 | 12·3 | 13·2 | 16·0 | 14·5 | 13·3 |
| 10 | 12·0 | 11·2 | 11·8 | 14·0 | 13·5 | 13·0 |

* Indicates plasma from a patient suffering from obstructive jaundice. The other specimens came from normal persons. 0·1 ml. of each component added at 37·5° C.

The inhibitory action of 0·025 M. calcium chloride on the coagulation of human plasma using rabbit's thromboplastin has already been pointed out by Stewart and Pohle (1938) and was confirmed by Quick (1942).

From Table 4 it is apparent that the same is true for the coagulation in the homologous system.

## DISCUSSION.

The greater susceptibility of man to haemorrhagic diseases has been correlated with the fact that shed human blood takes longer to clot than most mammalian blood. Whether this delayed clotting of human blood is due to a prothrombin deficiency or to slow convertibility into thrombin will be a matter of controversy until a direct method of estimation of prothrombin is available. However, for clinical purposes the behaviour of prothrombin in whole blood is of greater significance than the part played by the isolated components. Further, the exact experimental conditions under which the clotting time is determined are probably of no consequence provided that thromboplastin is present in excess and that the results obtained are expressed in relation to the clotting time of normal blood specimens from the same species determined under similar conditions. The situation is altered however, when plasma coagulation of different species is compared. Several workers have observed that shorter clotting times are obtained when plasma reacted with organ extracts from the same species and that heterologous organ extracts are inferior. Only a few workers have used human organs as the source of thromboplastin; Eley and co-workers (1936) report that placental extracts accelerate markedly the coagulation of recalcified human plasma and Edsall and workers (1944) using this preparation find that its activity is not quite so high as that of some thromboplastins of animal origin. Further it was found by Quick (1941) that thrombo-

[1] The statistics were calculated for those cases in which both thromboplastin preparations were used.

plastin isolated from human brain was inferior to rabbit's brain when tested with human plasma. Owen and Toohey (1941) find that either human or rabbit's brain may be used for plasma coagulation, but no indications of quality are given. From the results obtained in this communication it is apparent that human thromboplastin conforms to the general rule that thromboplastin shows greater activity when combined with homologous plasma. Thus thromboplastin from rabbits' tissue cannot be accepted as standard for comparative prothrombin estimations. A second factor which makes comparison of the prothrombin content of two species difficult is the varying influence of temperature on the systems. The results indicate that the temperature coefficient of the homologous human plasma-thromboplastin combination is lower than that of the homologous rabbit combination. These two factors tend to make the prothrombin concentration of human plasma appear lower than is actually the case. The third factor, the calcium concentration is difficult to evaluate in the estimation of prothrombin when oxalated plasma and different thromboplastin preparations are used, because calcium oxalate is not completely precipitated in the short time of the test and hence an uncontrollable amount of calcium ions is left in solution. In addition a certain amount of calcium is introduced by the thromboplastin. Calcium estimations in various thromboplastin preparations gave values of 17 to 29 mg. p.c. of soluble calcium in the dried brain powder. However Ransmeier and McLean (1938) have shown that the requirement for calcium ions in plasma coagulation differs from one species to the other.

For these reasons it is not possible accurately to express prothrombin content of one species in terms of another when Quick's technique is applied but it seems reasonable to estimate the efficiency of the normal human prothrombin system at between 30 and 40 p.c. of that of the rabbit.

## SUMMARY.

In the estimation of prothrombin using Quick's technique, thromboplastin prepared from human brain conforms to the general rule that thromboplastin shows greatest activity when reacting with its homologous plasma. The temperature coefficient of plasma coagulation when carried out in the presence of excess of thromboplastin is lower for human than for rabbit's plasma.

*Acknowledgment.* Appreciation is expressed to Dr. R. Willis, Pathologist of the Alfred Hospital, for the supply of human brains.

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# STAPHYLOCOCCAL FIBRINOLYSIN

by R. CHRISTIE, J. J. GRAYDON AND E. F. WOODS

(From the Commonwealth Serum Laboratories, Melbourne).

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The ability of staphylococci to lyse fibrin clot is shown by some of the pathogenic strains. The tube test to detect fibrinolysin production by streptococci (Tillett and Garner, 1933) cannot be used satisfactorily with staphylococci because of the length of time taken for lysis of the fibrin, the difficulty of reproducing results and the tendency of sterile clots to lyse. Christie and Wilson (1941) showed that agar plates made turbid with heat-precipitated fibrinogen could be used to detect lysis of fibrinogen by staphylococci and gave reasons for assuming that the agent which lysed heat-precipitated fibrinogen was fibrinolysin.

Aoi (1932) and Fisher (1936) obtained fibrinolysin in culture filtrates; Neter (1937) found that commercial staphylococcal antitoxin would inhibit fibrinolysis. Tests carried out here have confirmed and extended these observations as well as thrown some light on the nature of the Muller phenomenon (see below).

## EXPERIMENTAL.

*Production of Fibrinolysin.*

Strains of fibrinolytic staphylococci were grown in a modified Dolman's medium (Morgan and Graydon, 1936) for two days at 37° C. in an atmosphere of 80 p.c. oxygen and 20 p.c. carbon dioxide. The broth was then clarified by centrifugalization and 0·02 p.c. merthiolate added to prevent further growth of organisms. This procedure is similar to that used in the production of staphylococcal toxin.

*Titration of Fibrinolysin.*

Oxalated human plasma was heated for 5 minutes at 53–56° C. The precipitated fibrinogen was removed by centrifuging and re-suspended in a volume of merthiolated saline equal to that of the discarded supernatant fluid. Serial doubling dilutions of the fibrinolytic solution were made with merthiolated saline. Sixteen drops of each dilution were transferred to small test tubes, 2¼″ × ⅝″, four drops of the fibrinogen suspension were added and the tubes were placed in a water bath at 37° C. Lysis of the fibrinogen was apparent in the more concentrated solutions after four hours and final readings were made after 18 to 24 hours. In a typical titration complete lysis occurred at 80- to 160-fold dilution, with partial lysis at a 320- to 640-fold dilution. Sheep and guinea-pig fibrinogen gave similar end-points but the lysis in the more concentrated solutions occurred earlier than with human fibrinogen.

It seemed possible that fibrinogen in solution would be more susceptible to attack than in the precipitated form. A fibrinogen solution was therefore prepared from plasma by salting out the fibrinogen with 50 p.c. saturated sodium chloride solution and re-dissolving it, after washing, in distilled water. This solution was treated for 18 hours with serial dilutions of a culture filtrate prepared as above and the tubes then placed in a water bath at 53° C. to precipitate unaltered fibrinogen. Precipitates were obtained only in the tubes with higher dilutions of the filtrate, but the titre of the filtrate was similar to that obtained in a direct titration with heat-precipitated fibrinogen.

This result, coupled with the fact that heat-precipitated fibrinogen which has been lysed by a filtrate is no longer precipitable by heat, suggests that when heat-precipitated fibrinogen is lysed the change is not merely a form of disaggregation as has been suggested to occur when fibrin is lysed by staphylococcal filtrates (Birch-Hirschfeld, 1940).

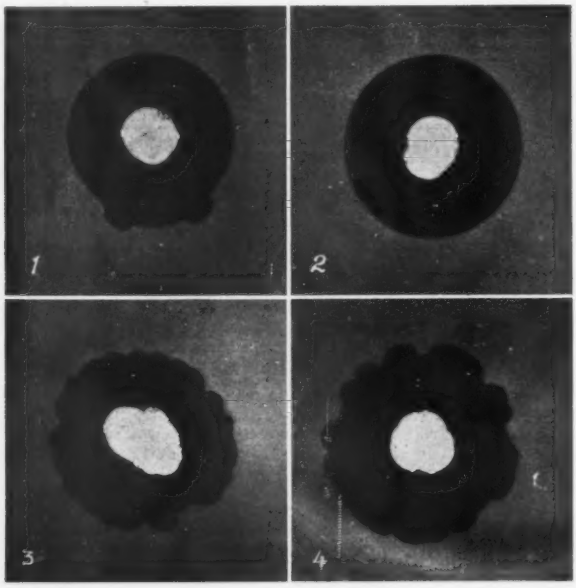
*Effect of Filtration and Heating.*

A broth culture was filtered through a Seitz E.K. filter, shown to be sterile and titrated against fibrinogen. The same end-point was reached as with unfiltered broth culture. Samples of the filtrate were heated for 5, 10, 15 and 30 minutes at 60° C., and for 5 minutes at 100° C. Slight precipitates caused by the heating were removed by centrifuging. No loss in fibrinolysin

was detected on titrating the samples thus obtained. Fibrinolysin is evidently much more heat-stable than α toxin. Its ability to pass through an asbestos filter-pad differentiates it from coagulase.

*Connection with the Staphylococcal Haemotoxins.*

Most pathogenic animal strains are non-fibrinolytic (Madison, 1935–6; Christie and Wilson, 1941). These strains usually produce much β toxin and since the few strains tested here which were fibrinolytic (4 out of 43) were those which produced little or no β toxin, it seemed possible that β toxin was inhibiting the action of the fibrinolysin produced by the other strains. It has been shown that β toxin inhibits the haemolysis of sheep red cells by α toxin (Christie and Graydon, 1941).




**Figs. 1–4.** Photographs of four colonies of staphylococci on fibrinogen agar, one showing a regular outline to the zone of lysis and the other three showing irregular outlines.
Photographs by Mr. F. J. Dempster.

However, when a drop of strong β toxin was added to each tube in a fibrinolysin titration row, no change in titre was obtained although the rate of lysis was reduced.

A suspension of heat-precipitated fibrinogen was then treated overnight with strong β toxin and used in the titration of a culture filtrate. The rate of lysis was reduced but the final end-point was the same as that obtained in a parallel titration using fibrinogen not previously treated with β toxin.

A strain producing much β toxin but showing no fibrinolysin on fibrinogen agar plates was grown in broth for two days. The filtrate showed no lytic activity towards fibrinogen either before or after neutralizing the β toxin with β antitoxin.

In the early part of this work it was suspected that α toxin and fibrinolysin were identical but this was discounted by their different susceptibilities to heat, the results of the neutralization tests (see below) and finally by the fact that it was possible to produce a filtrate from one strain (839) with a high α toxin content but no detectable fibrinolysin.

*Neutralization Tests.*

Constant volumes of a culture filtrate were treated for 30 minutes with various dilutions of staphylococcal α antitoxin to determine whether fibrinolysin was neutralized by α antitoxic serum. Serum dilutions in 10 p.c. stages were used. An antitoxin, P31, containing 90 inter-

national units per ml. and a concentrate of this, R33, containing 240 units per ml. were used. The antitoxic content was determined by reference to standard serum containing 20 units per ml. R33 showed approximately 2½ times as much neutralizing power for fibrinolysin as that possessed by P31, the anti-fibrinolytic power being parallel to the α antitoxin content. The anti-fibrinolytic factor and the antitoxic factor were either identical or had been precipitated during concentration in the same pseudo-globulin fraction.

A sample of standard serum showed less than a tenth of the neutralizing power for fibrinolysin possessed by P31 serum although the antitoxic titres of the two were in the ratio of 1: 4½. The standard serum is a purified product. Since this might have contained glycerine or borate buffer, the titration was repeated with preparations of the other sera to which glycerine and borate buffer had been added but no change in anti-fibrinolytic titre was obtained. Gengou (1933) showed that albumin and globulin influenced fibrinolysis. Normal horse serum which showed no anti-fibrinolytic activity was therefore added to standard serum but still with no effect on its neutralizing power for fibrinolysin.

It was therefore evident that α antitoxin was not the agent responsible for neutralizing the fibrinolysin but that fibrinolysin was antigenic and that immunization of horses with staphylococcal filtrates could produce an anti-fibrinolysin.

*Anti-fibrinolysin in Human Serum.*

Four samples of human serum (obtained through the courtesy of Miss H. Butler) were titrated for their anti-fibrinolytic ability as follows: Tubes containing one drop of a filtrate capable of causing lysis in a 40-fold dilution and 16 drops of dilutions of human serum were left at 37° C. for 30 minutes; 3 drops of fibrinogen suspension were then added to each tube and the whole left overnight at 37° C. The highest dilution of serum capable of inhibiting lysis was then noted. Two samples which were from people convalescent from severe infections with fibrinolytic staphylococci gave titres of 1/10 and 1/20. Two others from normal people gave titres of 1/5 and 1/10. There was thus no reason to suspect that recent infection gave a significantly high anti-fibrinolytic content in serum.

*The Muller Phenomenon.*

Muller (1927) reported that when certain staphylococci were grown on human blood agar plates in a continuous film for several days, discrete zones of clearing could be seen from the under side of the plates. Burnet (1928) showed that the same phenomenon could be seen around colonies of staphylococci on human blood agar where it manifested itself as small discrete zones of apparent lysis beyond the main circular zone of haemolysis. Packalen (1941) showed that the reaction was one of haemodigestion and not of haemolysis since it showed better on lysed blood agar than on blood agar. All agreed that a thermolabile substance in serum was essential for the production of the discrete zones. Another manifestation of the same reaction shown by Packalen was the appearance of zones of digestion of serum, shown as small discrete pittings in the medium around colonies of staphylococci on inspissated serum plates to which unheated serum had been added.

We observed another form of the phenomenon with fibrinolytic staphylococci grown on fibrinogen agar plates. The periphery of the zones of clearing around the colonies were frequently irregular (see photographs), and small discrete zones of lysis were occasionally found outside the main zones. A similar observation has been made by Fisk and Mordvin (1943). With some samples of medium, zones of lysis with outlines in the shape of irregular polygons, some even with re-entrant angles, were obtained. A strain which produced very little fibrinolysin occasionally gave only small discrete zones, one, two or three, adjoining the colony, without the complete zone shown by more strongly fibrinolytic strains.

An examination of 79 strains of staphylococci (21 non-pathogenic and non-fibrinolytic, 37 pathogenic and fibrinolytic, 21 pathogenic and non-fibrinolytic) showed that all of the 37 fibrinolytic strains could produce the Muller effect on human lysed blood agar while none of the nonfibrinolytic strains were able to do so.

These figures, together with the appearances of the discrete zones on fibrinogen agar plates, led us to the assumption that fibrinolysin was the agent responsible for the Muller phenomenon and that the irregularities on the plates were another manifestation of the phenomenon.

Support for this assumption was got from the fact that a thermolabile substance in serum is necessary for the production of the irregularities on the plates, just as it is for the discrete zones on human blood agar plates. If fibrinogen is heat-precipitated, centrifuged off, re-suspended in saline and used to prepare plates, the zones of lysis around staphylococcal colonies have perfectly circular peripheries. The irregularities on fibrinogen agar are not obtained if the fibrinogen is precipitated by prolonged heating of the plasma (30 minutes) at 56° C.; a medium thus prepared gives extensive zones with circular peripheries. Heating at 53° C. for three minutes gives a medium which shows the irregularities and less extensive zones. Two plates of fibrinogen agar were prepared in which the medium had been heated for 30 minutes at 56° C. Before pouring, 2 ml. of unheated serum were added to one of them. Fibrinolytic staphylococci

produced zones of lysis around their colonies with marked irregularities only on the medium containing the unheated serum.

The appearances suggested that the part played by the thermolabile substance was an inhibitory one, its antagonistic action being such as to prevent lysis by the staphylococcal agent. The well-defined edges of the zones of lysis, which were less marked when the inhibitory substance in the serum had been destroyed by heat, indicate that there was a fairly critical concentration below which the lysin could not act in the presence of unheated serum. Where lysis occurs in discrete areas beyond the general zone of lysis, local conditions must be favourable and the most probable assumption is that adsorbent particles act there either by concentrating the lytic agent or by removing the inhibitory one. Burnet found that in the centre of the Muller zones on blood agar a particle could sometimes be seen. We occasionally found such particles in lysed zones and significance was imparted by the fact that, when present, they were always centrally placed. The absence of visible particles in many zones where lysis had occurred did not necessarily prove that they were not present originally.

The solution of Muller's problem has not yet been achieved but it seems probable that the explanation of the discreteness will be found along physical rather than chemical lines.

## SUMMARY.

A method is described for detecting and titrating staphylococcal fibrinolysin in broth cultures.

Fibrinolysin will pass through a bacterial filter. It is thermostable and antigenic.

Antifibrinolysin can be detected in human serum and in the sera of immunized horses.

Fibrinolysin is the agent responsible for the Muller phenomenon. A new manifestation of this phenomenon is described.

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# THIOL—VITAMIN K MECHANISM IN THE CLOTTING OF FIBRINOGEN

by R. N. LYONS

(Kanematsu Institute of Pathology, Sydney Hospital).

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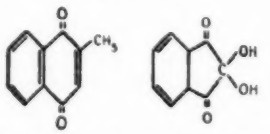
Since vitamin K was identified in 1935, studies upon its mode of action have only revealed that it is essential for the formation of prothrombin, and that this synthesis of prothrombin occurs in the liver (Smith, *et al.* 1937). An early suggestion that vitamin K might be a prosthetic group in the prothrombin molecule was discounted on the grounds that large amounts of prothrombin failed to relieve the haemorrhagic lesions in vitamin K deficient chicks when mixed with their diet (Dam, *et al.* 1938).

Later it was suggested that in view of the ability of vitamin K to oxidize thiol groups, the synthesis of prothrombin could occur by uniting proteins through their thiol groups and forming disulphide linkages (Bernheim and Bernheim, 1940). Whilst this may or may not be so, experiments performed in this laboratory suggest that vitamin K could be an integral and functional part of the prothrombin molecule.

Since the quinone structure is characteristic of vitamin $K_1$, the report of Chargaff and Ziff (1941) that "ninhydrin" (triketohydrindene hydrate) would clot fibrinogen was significant. The structural relationship between ninhydrin and 2–methyl–1 : 4–naphthoquinone is apparent.




2–methyl–1 : 4–naphthoquinone "Ninhydrin"

The same workers also tried synthetic vitamin K derivatives and found them inactive. Their experiments were very unfortunate, however. They used first an aqueous solution of 2–methyl–1 : 4–naphthoquinone which is negligibly soluble in water. Secondly, they tried the water soluble 2–methyl–1 : 4–naphthohydroquinone-diphosphate, which has both its keto groups reduced and esterified, and requires enzyme action followed by oxidation to convert it into 2–methyl–1 : 4–naphthoquinone. In a later paper (Chargaff and Bendich, 1943) they reported that other substances, including naphthoquinone derivatives were able to coagulate fibrinogen, but again denied that vitamin K had this property on the grounds that two sulphonic acid derivatives of naphthoquinone which have vitamin K activity, did not clot fibrinogen. It had recently been shown (Richert, 1944) that all active vitamin K preparations which have not the free quinone structure are converted to the quinone form *in vivo*, and are excreted in a combined form.

Experiments in this laboratory have shown that 2–methyl–1 : 4–naphthoquinone can form a solid gel with specially prepared fibrinogen and that this gel is indistinguishable microscopically from a thrombin clot. It must be stressed, however, that the fibrinogen must be specially prepared. If fibrinogen is pre-

pared from fresh plasma by repeated precipitation with sodium chloride it will not gel with 2–methyl–1 : 4–naphthoquinone, nor with "ninhydrin." To this can be attributed the failure of Ferguson and Ralph (1943) to reproduce the results of Chargaff and Ziff (1941) with ninhydrin clots.

Further experiments have shown that 2–methyl–1 : 4–naphthoquinone acts by oxidizing the thiol groups of fibrinogen prepared from aged, sterile plasma which has had a preliminary treatment with calcium phosphate, producing an immediate gel. In order to avoid confusion this fibrinogen will be called fibrinogen B, whereas the fibrinogen prepared from fresh plasma by precipitation with sodium chloride only, will be called fibrinogen A. It has already been suggested (Baumberger, 1941) that a thiol mechanism appears to be involved in blood clotting. It has also been shown that 4–amino–2–methyl–naphthol which is oxidized to the quinone form in air can oxidize thiol groups to disulphide linkages (Bernheim and Bernheim, 1940).

There have been some reports (Larson and Greenberg, 1936; Glazho and Greenberg, 1939) of a diffusible organic substance which accelerates blood clotting. Experiments described in this paper show that 2–methyl–1 : 4–naphthoquinone greatly accelerates blood clotting, as also do organic disulphide compounds, aromatic amines and peroxidase.

Experiments on the preparation of fibrinogen B showed that it is most readily produced from aged, sterile, citrated plasma which has had solid tricalcium phosphate added to it. A recent paper (Quick, 1943) suggests that prothrombin is composed of two components united through calcium, one of the components disappearing on standing. Whether one of these components is responsible for the conversion of fibrinogen A into fibrinogen B is at present being investigated. However, further experiments show that at least two changes are involved when thrombin reacts with fibrinogen, the first liberates thiol groups and the second is an oxidation of thiol groups which may be brought about by a naphthoquinone or a naphthoquinone-protein complex. The product of this oxidation is a fibrin gel.

Evidence in support of this is presented in the following order:

(1) Polarographic estimation shows a relatively large number of thiol groups in fibrinogen B.
(2) Similar estimations show relatively few in fibrinogen A.
(3) Addition of dilute thrombin to fibrinogen A increases the number of thiol groups.
(4) Fibrinogen B will gel immediately upon the addition of 1·0 $\mu$g. of 2–methyl–1 : 4–naphthoquinone in alcoholic solution per ml.
(5) Blood clotting is greatly accelerated by 2–methyl–1 : 4–naphthoquinone.
(6) Thrombin digested with trypsin gives a colour reaction characteristic for naphthoquinone with 2 : 4–dinitrophenyl-hydrazine and with ethyl cyanoacetate.
(7) The clotting of fibrinogen A by thrombin can be greatly accelerated by addition of peroxidase and aromatic amines.
(8) Fibrinogens A and B both catalyse the iodine-azide reaction.
(9) Fibrin is readily soluble in sodium sulphide (which disrupts –S–S– linkages).
(10) Blood clotting can be inhibited by an excess of thiol compounds and by the substances which react with thiol groups, (a) Mercury compounds. (b) Arsenic compounds of the arsenoxide type. (c) Cyanides.
(11) The immediate oxidation of thiol compounds by 2–methyl–1 : 4–naphthoquinone can be shown polarographically.

## EXPERIMENTAL.

*Preparation of Reagents.*

*Fibrinogen A.* Blood is taken as quickly as possible into isotonic sodium and potassium oxalate with continued mixing to minimize any formation of thrombin during the collecting. After centrifuging 1·2 volumes of saturated sodium chloride are added, the precipitated fibrinogen is obtained by running off the saline-plasma in a separating funnel. It is dissolved in one-third of its original volume of oxalated saline. This precipitation is repeated twice more. The final product is a water clear solution. If filtration is necessary it is filtered through sintered glass, not filter paper, as the calcium salts in filter paper can convert it into fibrinogen B, especially in the early stages of its precipitation, when a small amount of prothrombin is present.

*Fibrinogen B.* 100 ml. of aged citrated plasma are mixed with 5 gm. tricalcium phosphate and stirred at frequent intervals for 1 hour. After centrifuging 1·2 volumes of saturated sodium chloride are added for each volume of supernatant plasma. The precipitated fibrinogen is obtained as above. The fibrinogen is dissolved in 50 ml. 0·85 p.c. sodium chloride and the precipitation with saturated sodium chloride repeated twice more. Finally the fibrinogen is dissolved in 35 ml. 0·85 p.c. sodium chloride, centrifuged if necessary, and stored in the refrigerator under an atmosphere of hydrogen. The solution should be water clear, and is quite stable for one week under hydrogen.

The plasma used was obtained from citrated human blood which had been kept for over two weeks and was no longer useful for blood transfusion. It contained 200 ml. of 3·0 p.c. sodium citrate and 40 ml. of 15 p.c. glucose per litre. The blood was collected into the glucose-citrate which had a pH of 7·5.

*Thrombin.* 100 ml. of oxalated plasma are diluted 12 times with distilled water, pH is taken to 5·3 with 2 p.c. acetic acid and the precipitate separated by centrifuging. The precipitated prothrombin and fibrinogen are dissolved in 20 ml. 0·85 p.c. sodium chloride, 1 ml. of thromboplastin suspension is added and the pH adjusted to 7·4. 1 ml. of M/40 calcium chloride is added and the solution stirred for a few minutes. The clot forms round the stirring rod and is removed.

This solution when diluted approximately 100 times will clot an equal volume of plasma in 15 secs. at 37° C.

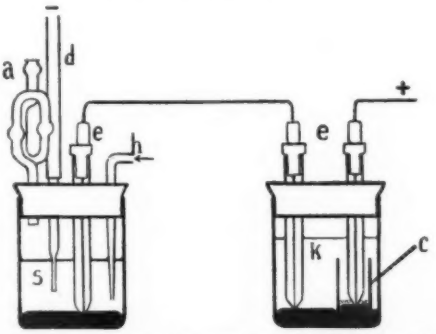



**Fig. 1.** Dropping mercury electrode and calomel half-cell. a, hydrogen exit; c, calomel cell; d, dropping mercury electrode; e, platinum wire electrode joining mercury pools; h, hydrogen inlet; k, saturated potassium chloride solution; s, solution being investigated.

*Thromboplastin.* Thromboplastin is prepared by drying with acetone rabbit brain which has been freed from blood vessels and membrane. The powdered brain can be kept for many months under hydrogen. The solution of thromboplastin used is made by suspending 0·1 gm. of dry brain in 10 ml. 0·85 p.c. sodium chloride, heating to 56° C. for 10 minutes, then standing for 15 minutes and pipetting off the supernatant liquid. The supernatant liquid will clot normal oxalated plasma in presence of $Ca^{++}$ in 12 sec. All clotting experiments are carried out in a thermostat at 37° C.

*2–methyl–1: 4–naphthoquinone.* 10 mg. of 2–methyl–1: 4–naphthoquinone are dissolved in 50 ml. alcohol, 10 ml. phosphate buffer pH 7·5 and 40 ml. water.

*Phosphate buffer.* Primary: 9·078 gm. of potassium dihydrogen phosphate per litre. Secondary: 23·8 gm. of disodium hydrogen phosphate, (12 $H_2O$) per litre. A mixture of primary to secondary in the ratio of 4: 1 gives a pH 7·4. Other proportions can be used depending upon the nature of the substance being dissolved.

*Plant peroxidase.* Pulped potato is freed from starch by kneading in a muslin bag under water. When starch-free it is further kneaded in a fresh 200 ml. of water for 10 minutes. This extract contains a mixture of phenoloxidases and peroxidase.

*Iodine solution (N/10).* 20 gm. of potassium iodide are dissolved in 30 ml. water, 12·7 gm. iodine are then added and dissolved. The solution is then diluted to 1 litre with water.

*Iodine-sodium azide solution.* 3 gm. of sodium azide are dissolved in 100 ml. of N/10 iodine.

*Thioglycollate.* 1 gm. of thioglycollic acid is titrated with 10 p.c. sodium carbonate to pH 7·4. It is then diluted with phosphate buffer pH 7·4 to a concentration of 5 gm. per 100 ml. This solution is not stable in air, and should be made up freshly or kept under hydrogen.

*Anticoagulant solution.* 450 ml. of blood are taken into 10 ml. of oxalate solution containing 0·3 gm. sodium oxalate and 0·372 gm. potassium oxalate and which has previously had the pH adjusted to 7·5.

*pH control and determinations.* The pH of every solution was checked with a Leeds and Northrop pH meter (glass electrode, absolute accuracy ± 0·05 pH unit).

*Polarographic analysis.* The polarographic analysis cell was specially designed to avoid diffusion in order that the work could be carried out on a micro scale. The pool of quiet mercury in the bottom of the cup was directly connected by platinum wire to another mercury pool covered with saturated potassium chloride, and this saturated potassium chloride acted as a salt bridge connecting with a saturated calomel electrode (see Fig. 1). Regarding the general polarographic method, see e.g. Kolthoff and Lingane (1941).

Polarographic experiments were conducted in a thermostat at 25° C.

## RESULTS.

### *Polarographic Determination of Thiol Groups.*

The concentration of thiol groups in fibrinogen was measured by a manually operated polarograph using a supporting electrolyte consisting of 0·001 N cobaltous chloride, 0·1 N ammonia and 0·1 N ammonium chloride, 0·1 ml. of a saturated solution of methyl cellulose in water being added to suppress the occurrence of maxima in the curves.

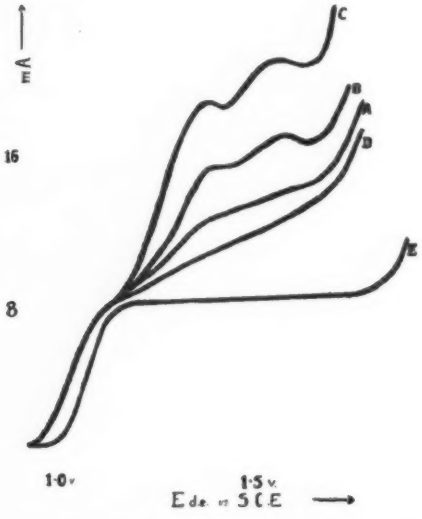



**Fig. 2.** Concentration of thiol groups in fibrinogen B. Ordinate, current in microamps; abscissa applied potential in volts between the dropping electrode and the calomel half-cell.

A, 0·3 mg. fibrinogen B per 100 ml.
B, 0·9 mg. fibrinogen B per 100 ml.
C, 1·5 mg. fibrinogen B per 100 ml.
E, supporting electrolyte.

Curve D represents a protein effect of fibrinogen A (0·3 mg. per 100 ml.) which can be distinguished from a typical thiol curve (fibrinogen B) by the absence of maxima.

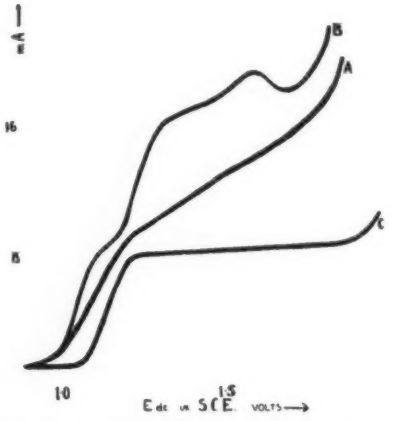



**Fig. 3.** Comparison of the concentration of thiol groups in fibrinogens A and B.

A, 0·5 mg. fibrinogen A per 100 ml.
B, 0·5 mg. fibrinogen B per 100 ml.
C, supporting electrolyte.

The fibrinogen solution was added to the ammonia–ammonium chloride buffer; hydrogen was passed through to free from air, and then the cobaltous solution was added and hydrogen passed again. Curve height was found to be proportional to the concentration of fibrinogen within the range used in these experiments. The three concentrations shown in Fig. 2 (0·3 mg., 0·9 mg. and 1·5 mg. of fibrinogen B per 100 ml.) giving the three curves, A, B and C respectively.

The number of thiol groups per mg. of fibrinogen is not constant. Fig. 3 represents an average result. However, fibrinogen B always has a higher concentration of thiol groups than

fibrinogen A. Usually the thiol groups of fibrinogen A are negligible, but occasionally their number may be as high as approximately half that found in fibrinogen B, which may be due to a partial formation of thrombin during the drawing of the blood or during the preparation of the fibrinogen.

Fig. 3 shows a typical comparison between fibrinogens A and B. The protein concentration is the same for both solutions, i.e. 0·5 mg. of protein per 100 ml. The protein concentration was estimated upon the total nitrogen value of the purified fibrinogen solutions.

*Reaction of Thrombin with Fibrinogen A at pH 9·5.*

With increasing alkalinity the clotting time of fibrinogen A with thrombin progressively increases. At pH 10 clotting is completely inhibited, but neutralization or the addition of excess 2–methyl–1: 4–naphthoquinone will produce an immediate gel.

The thiol groups of fibrinogen A were estimated before and after the addition of thrombin.

Fig. 4 shows the increase in number of thiol groups when 0·1 ml. of thrombin is added to fibrinogen A solution (0·5 mg. per 100 ml.) in a supporting electrolyte of 0·001 N cobaltous chloride, 1 N ammonia and 1 N ammonium chloride. Although the same increase was noted in 0·1 N ammonia-ammonium chloride, there was a slight precipitation of protein as shreds of clot formed. In N ammonia-ammonium chloride there is no precipitation during the time of the experiment.

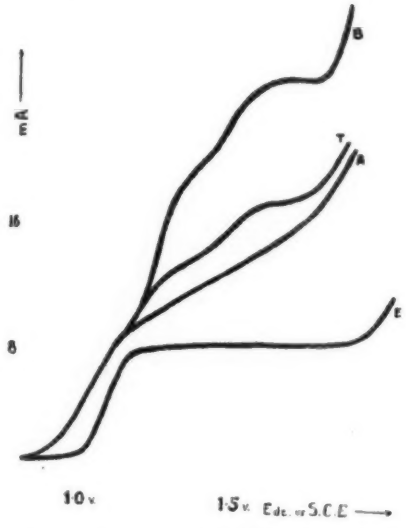



Fig. 4. Increase in thiol groups of fibrinogen A upon the addition of small amounts of thrombin.

A, 0·5 mg. fibrinogen A per 100 ml.
B, 0·1 ml. dilute thrombin added to A.
T, 0·1 ml. dilute thrombin.
E, supporting electrolyte.

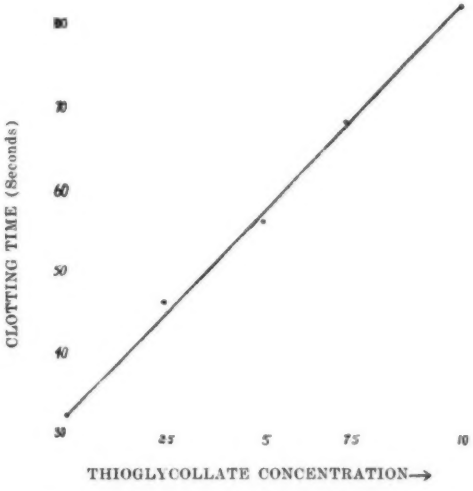



Fig. 5. Effect of sodium thioglycollate on thrombin-fibrinogen A solutions.

*Clotting of Fibrinogen B with 2–Methyl–1: 4–Naphthoquinone.*

Fibrinogen B solution forms a solid gel immediately an alcoholic solution of 2–methyl–1: 4–naphthoquinone is added, less than 1·0 μg. per ml. of fibrinogen solution being necessary. The gel formed is indistinguishable from that produced by thrombin when examined in the fresh state with dark ground illumination and using a magnification of 900 diameters.

1 ml. fibrinogen B + 1·0 μg. 2–methyl–1: 4–naphthoquinone in alcohol-phosphate buffer. Immediate gel.

Control 1 ml. fibrinogen B + alcohol-phosphate buffer. No gel.

*p*–Benzoquinone in aqueous solution also produces a solid gel with fibrinogen B, but this requires about 5 minutes and a higher concentration of benzoquinone (1 mg. per 10 ml.).

Fibrinogen A will not gel with 2–methyl–1: 4–naphthoquinone nor *p*–benzoquinone.

*Effect of 2–Methyl–1: 4–Naphthoquinone on the Reaction Between Thrombin and Fibrinogen A.*

At least two reactions are involved when thrombin reacts with fibrinogen A.

The first involves the sulphur groups of the fibrinogen and it is inhibited by heparin, sodium thioglycollate, reduced glutathione, alcohol, cyanides, arsenates and mercury compounds.

The second involves the oxidation of thiol groups liberated by the first reaction and is accelerated by 2–methyl–1: 4–naphthoquinone, peroxidase and aniline. These last two appear to act through a naphthoquinone complex in thrombin.

Since the alcohol in which the 2–methyl–1: 4–naphthoquinone is dissolved inhibits the first of these reactions, the naphthoquinone cannot be added before the thrombin, but is added afterwards. Studies upon the kinetics of the first reaction show that it is completed in approximately one-tenth of the normal clotting time, so that if thrombin and fibrinogen A take 40 seconds to clot and a solution of 2–methyl–1: 4–naphthoquinone is added after 4 seconds, an immediate clot forms. By adding the naphthoquinone at 2 second intervals to a series of tubes containing the same proportion of thrombin and fibrinogen A it is possible to determine the minimum time for the first reaction between fibrinogen A and thrombin.

By using this method the inhibition of the first reaction by excess thiol groups was studied.

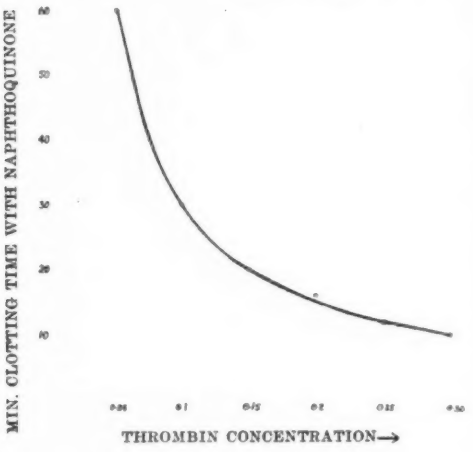



**Fig. 6.** Minimal clotting time of fibrinogen A with 2–methyl–1: 4–naphthoquinone and varying concentrations of thrombin.

The addition of thioglycollate or reduced glutathione before thrombin has a marked inhibitory effect on clotting, but if added shortly after, it has little or no effect.

| Control: | Min. C.T. with naphthoquinine. |
|---|---|
| 1 ml. fibrinogen A + 0·2 thrombin (dil. 1 in 100) | 32 sec. |
| 1 ml. „ + 0·1 ml. 2·5 p.c. thioglycollate + 0·2 ml. thrombin | 46 sec. |
| 1 ml. „ + 0·1 ml. 5·0 p.c. thioglycollate + 0·2 ml. thrombin | 56 sec. |
| 1 ml. „ + 0·1 ml. 7·5 p.c. thioglycollate + 0·2 ml. thrombin | 68 sec. |
| 1 ml. „ + 0·1 ml. 10·0 p.c. thioglycollate + 0·2 ml. thrombin | 82 sec. |
| 1 ml. „ + 0·2 ml. thrombin + 0·1 ml. 10 p.c. thioglycollate (added after 3 secs.) | 34 sec. |

Thus, by a series of experiments it is possible to study the kinetics of the first reaction. Serial dilutions of thrombin are added to fibrinogen and the minimal clotting time with 2–methyl–1: 4–naphthoquinone is recorded. It was found that the rate of reaction was directly proportional to the thrombin concentration, as was to be expected.

Control: 1 ml. fibrinogen A + 0·25 ml. thrombin (dil.) C.T. 150 sec.

| | Min. C.T. with 2–methyl–1: 4–naphthoquinone. |
|---|---|
| 1 ml. fibrinogen A + 0·3 ml. thrombin (dil.) | 10 sec. |
| 1 ml. „ + 0·25 „ „ + 0·05 ml. saline | 12 „ |
| 1 ml. „ + 0·20 „ „ + 0·10 „ „ | 16 „ |
| 1 ml. „ + 0·15 „ „ + 0·15 „ „ | 20 „ |
| 1 ml. „ + 0·10 „ „ + 0·20 „ „ | 30 „ |
| 1 ml. „ + 0·05 „ „ + 0·25 „ „ | 60 „ |

*The Presence of a Naphthoquinone Derivative in Thrombin.*

By using 2: 4–dinitrophenylhydrazine as a reagent for naphthoquinone as described by Novelli (1941) it was possible to demonstrate the presence of small amounts of naphthoquinone in thrombin and plasma. None could be shown in purified fibrinogen, or serum albumin solutions.

This reaction is very delicate and depends upon the development of a green colour which as far as is known, is only given by naphthoquinone and its derivatives.

If the thrombin was digested with trypsin at pH 8·0 for several hours, and extracted several times with small quantities of ether, and the ether extract concentrated, a strong green colour was observed upon applying Novelli's reaction.

100 ml. of thrombin solution were digested with 1 gm. of trypsin, at pH 8·0, for 6 hours at 37° C. The mixture was then extracted with three lots of 50 ml. of ether and the ether evaporated by passing a stream of air through it; this simultaneously oxidized any reduced naphthoquinone present. The residue was taken up in 3 ml. of alcohol and Novelli's test for naphthoquinone was applied.

A control experiment was carried out simultaneously using 1 gm. of trypsin in 100 ml. of thrombin at pH 8·0, which was extracted with ether before digestion and treated as above.

Control. Slight yellow-green colour.
Thrombin. Bright green colour.

To another portion of the alcoholic solution obtained from the ether residue, the reaction with ethyl cyanoacetate was investigated.

To 1 ml. of the alcoholic solution of the residue from the ether extract, 3 drops of ethyl cyanoacetate were added, followed by 5 ml. of borate buffer pH 10·5, and then allowed to stand for 10 minutes.

Control. Colourless.
Thrombin. Light blue colour.

This colour reaction of Craven (1931) for quinones has recently been used quantitatively by Richert (1944) for the determination of 2–methyl–1: 4–naphthoquinone in the urine.

*Acceleration of Clotting by Peroxidase and Aniline.*

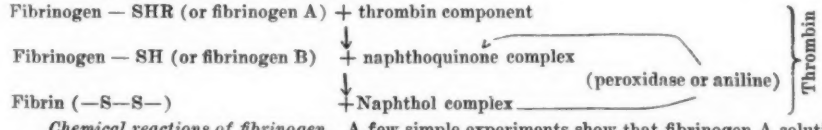
Plant peroxidase greatly accelerates the clotting of purified thrombin and fibrinogen A solutions. Aniline has a similar effect. Both these substances do not inhibit the first reaction between thrombin and fibrinogen, and can be added before thrombin.

| | C.T. |
|---|---|
| 1 ml. fibrinogen A + 0·1 ml. thrombin (dilute) | 180 sec. |
| 1 ml. „ + 0·1 ml. peroxidase + 0·1 ml. thrombin | 35 „ |
| 1 ml. „ + 0·01 ml. aniline emulsion + 0·1 ml. thrombin | 30 „ |

Peroxidase will not clot fibrinogen B nor convert fibrinogen A to fibrinogen B, hence it must act by oxidizing a phenolic group in thrombin. Since the presence of naphthoquinone in thrombin was suggested by the colour reactions described above, it could be assumed that peroxidases oxidize the naphthol formed when naphthoquinone reacts with the thiol groups of the fibrinogen.

The peroxidase activity of plasma and blood is so great that the addition of plant peroxidase to plasma and thrombin does not appreciably decrease the clotting time.

The reaction between fibrinogen A and thrombin could be represented thus (fibrinogen — SHR representing a blocked thiol group):


Fibrinogen — SHR (or fibrinogen A) + thrombin component
Fibrinogen — SH (or fibrinogen B) + naphthoquinone complex
(peroxidase or aniline)
Fibrin (—S—S—) + Naphthol complex
Thrombin

*Chemical reactions of fibrinogen.* A few simple experiments show that fibrinogen A solution has slight reducing properties and its clotting property is destroyed by oxidation.

*Oxidation of fibrinogen.* 5 ml. fibrinogen A solution were oxidized with a few drops of 2 p.c. potassium permanganate-magnesium sulphate mixture at pH 7·1. Whilst the fibrinogen solution appeared unaltered, it would no longer clot with thrombin. Oxidation of fibrinogen B solution with permanganate produces an amorphous precipitate. Apparently the sulphur is so combined in fibrinogen A that it can be further oxidized without causing any precipitation. Of course, excess permanganate caused a heavy precipitation of protein.

*Reaction of Fibrinogens A and B with Iodine-Azide Solution.*

The reaction which is catalysed by — SH and blocked — SH groups is $2NaN_3 + I_2 \rightarrow 2NaI + 3N_2$. Its rate can be measured by the volume of nitrogen evolved over a certain period or the time to the complete disappearance of free iodine, using starch solution as an indicator.

If the latter method is used the iodine-azide solution should be diluted to a suitable working strength.

Fibrinogen A before and after it has been treated with iodine reacts readily with iodine-azide solution. Therefore, the sulphur cannot be combined in an —SH linkage or a —S—S— linkage, because —SH is oxidized by iodine treatment to —S—S— which does not react. Hence it is suggested that the sulphur is protected in fibrinogen A in much the same manner as proposed by Potter and Du Bois (1943) for their succinic-dehydrogenase system. All the reactions and properties of fibrinogen A described in this paper are typical of a blocked protein —SH bond which is later converted to a protein —SH (fibrinogen B), which is oxidized by a thrombin component (possibly a naphthoquinone complex) to a protein —S—S— protein form; large numbers of these linkages would give the typical fibrin structure.

Fibrinogen B gives a vigorous reaction with the iodine-azide solution before oxidation with iodine, but after such oxidation gives only a very weak reaction. Immediately iodine solution is added to it, a tenuous clot forms, whereas fibrinogen A is unaffected.

Thrombin gives the iodine-azide reaction more vigorously than fibrinogen A before oxidation, but not as vigorously after both have been oxidized

Tests before oxidation were carried out in the following manner; 5 ml. of protein solution to be tested were added to 1 ml. of iodine-azide solution and the rate of nitrogen evolution observed.

| | Rate of reaction. |
|---|---|
| Fibrinogen A | + + |
| Fibrinogen B | + + + |
| Thrombin | + + + |

Oxidation with iodine was carried out by adding 1 ml. of N/10 iodine solution and 1 ml. of acetate buffer (pH 5·8) to 5 ml. of the solution to be tested and warming to 50° C. for 10 minutes.

The rate of the iodine-azide reaction was observed as before, 1 ml. of iodine-azide being added to the oxidized solutions.

| | Rate of reaction. |
|---|---|
| Fibrinogen A | + + |
| Fibrinogen B | — |
| Thrombin | + |

*Solubility of Fibrin in Sodium Sulphide.*

Fibrin is readily soluble in sodium sulphide at pH 7·5. A clot will dissolve in 10 p.c. sodium sulphide in a few minutes. This suggests the presence of disulphide linkages which are disrupted by sodium sulphide, thus:

$$R.S - S.R_1 + 2Na_2S \rightarrow R.S\,Na + R_1S\,Na + Na_2S_2.$$

*Action of Substances Reacting with Thiol Groups and of Excess Thiol Groups.*

Fildes (1940) has shown that mercury compounds can inhibit biological thiol mechanisms. Both fibrinogens A and B completely lose their clotting properties when treated with organic mercurials.

Merthiolate (sodium ethyl mercuri-thiosalicylate) one of the least ionized organic mercurials will completely destroy the clotting ability of both fibrinogens A and B after some days, without causing any precipitation of protein.

*Control*: (a) 10 ml. of sterile fibrinogen A stored in a refrigerator for one month can still be readily clotted with thrombin.

(b) 10 ml. of sterile fibrinogen A, containing 1: 10,000 merthiolate, stored in a refrigerator for 7–14 days completely loses its clotting activity with thrombin.

(c) 10 ml. of sterile fibrinogen B, containing 1: 10,000 merthiolate, stored in a refrigerator for 7 days completely loses its clotting activity with thrombin.

*Effects of excess thiol groups on thrombin.* 5 ml. of thrombin solution were well mixed with 3 ml. of 4 p.c. sodium thioglycollate pH 7·3 in a centrifuge tube and allowed to stand overnight. 8 ml. of acetone were added, the precipitated thrombin was centrifuged and well washed with water at pH 5·3. It was then taken up in 5 ml. of saline at pH 7·4. This thrombin will no longer clot fibrinogen A. It appears that this reaction of thrombin with excess thiol compounds is irreversible as attempts to regenerate active thrombin failed.

*Control*: 5 ml. of thrombin solution were well mixed with 3 ml. saline and treated as in previous experiment. The thrombin after acetone precipitation was re-dissolved and readily clotted fibrinogen A.

*Sodium cyanide and arsonates inhibit clotting.* 1 gm. of sodium cyanide is dissolved in 10 ml. of phosphate buffer and the pH is adjusted to 7·4.

| | C.T. |
|---|---|
| 1 ml. fibrinogen + 0·1 ml. phosphate buffer + 0·1 ml. dil. thrombin | 30 sec. |
| 1 ml. fibrinogen + 0·1 ml. cyanide + 0·1 ml. dil. thrombin | No clot up to 3 hrs. |
| 1 ml. fibrinogen + 0·1 ml. "acetylarsan" + 0·1 ml dil. thrombin | No clot up to 3 hrs. |

*The Oxidation of Thiol Compounds by 2–Methyl–1: 4–Naphthoquinone.*

By studying the reaction between thiol compounds and 2–methyl–1: 4–naphthoquinone polarographically, it can be seen that the reaction is instantaneous.

Immediately sodium thioglycollate or l-cysteine is added to the naphthoquinone, the characteristic wave of naphthoquinone disappears.

The anodic wave of the thiol compounds also decreases with successive addition of 2–methyl–1: 4–naphthoquinone.

This is shown in Fig. 7, where a M/1,000 solution of 2–methyl–1: 4–naphthoquinone is slowly added to M/1,000 solution of l-cysteine.

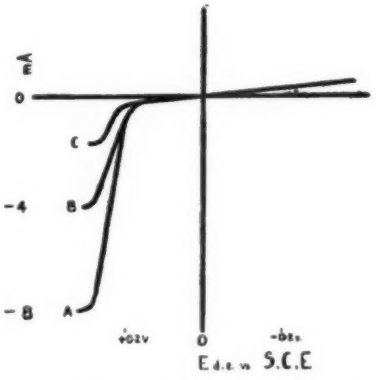



**Fig. 7.** Addition of 2–methyl–1: 4–naphthoquinone to l-cysteine solution.

A, anodic curve of M/1,000 l-cysteine in N/10 potassium nitrate.
B, addition of one-third volume of M/1,000 2–methyl–1: 4–naphthoquinone to A.
C, addition of one-half volume of M/1,000 2–methyl–1: 4–naphthoquinone to A.

## DISCUSSION.

Although it is known that vitamin K is necessary for the formation of prothrombin, the possibility that it is an integral part of prothrombin has been doubted because large amounts of dried prothrombin mixed with food failed to relieve the haemorrhagic lesions in K-avitaminous chicks.

Experiments described here suggest that vitamin K is actually a functional part of the thrombin molecule. At least two reactions appear to be involved when thrombin reacts with fibrinogen, and the clotting mechanism is dependent upon the sulphur linkages of the fibrinogen.

Polarographic estimations performed at intervals during clotting suggest that initially fibrinogen A contains many blocked protein-SH groups which are quickly converted by thrombin to protein-SH, this system being analagous to the thiol mechanism in succinic dehydrogenase suggested by Potter and Du Bois (1943). The protein –SH form of fibrinogen can be prepared from aged citrated plasma, and this type of fibrinogen B can be immediately converted by minute amounts of 2–methyl–1 : 4–naphthoquinone to the form protein –S–S– protein giving a clot. The clot thus formed is indistinguishable microscopically from the clot formed with thrombin, having a typical gel structure.

Conflicting reports of the ability of "ninhydrin" to clot fibrinogen could be due to the existence of these two forms of fibrinogen.

The presence of small amounts of a naphthoquinone derivative in thrombin has been suggested by two colour reactions which were applied to a tryptic digest of thrombin.

Although fibrinogen B is an intermediate between fibrinogen A and fibrin, it does not appear to be identical with profibrin (Hammarsten, 1879; Apitz, 1937). It was not found possible to produce fibrinogen B by either heating or freezing fibrinogen; a similar protein assumed to be profibrin was obtained.

SUMMARY.

Evidence is presented which suggests that vitamin K is a functional part of the thrombin molecule.

Clotting of fibrinogen by thrombin occurs in at least two stages; the initial step is the liberating of blocked thiol groups in fibrinogen brought about by one component of thrombin; the second, an oxidation, probably by a naphthoquinone complex in thrombin converting protein –SH to protein –S–S–protein (fibrin).

Quantitative estimations of the thiol groups of fibrinogen were made polarographically. Qualitative tests from which the presence of thiol groups and blocked thiol groups was deduced were: (1) Catalysis of the iodine-azide reaction. (2) The inhibition of clotting by inhibitors of thiol reactions. (3) The inhibition of clotting by excess of thiol compounds.

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# THE MORTALITY OF THE IMMATURE STAGES OF *CALANDRA ORYZAE* L. (SMALL STRAIN) AND *RHIZOPERTHA DOMINICA* FAB. IN WHEAT OF DIFFERENT MOISTURE CONTENTS

by L. C. BIRCH[1]

(Waite Agricultural Research Institute, University of Adelaide).

*(Accepted for publication 24th November, 1944.)*

It is well known that the development of an infestation of insects in stored wheat may be limited by the dryness of the wheat. There is, however, little information available defining precisely the critical level of dryness for the different insects. Existing information is summarized by Davidson (1941) and Ratcliffe, Gay and Fitzgerald (1940). The influence of dryness on the mortality of the eggs of *C. oryzae* and *R. dominica* has been described elsewhere (Birch, 1944). The influence of temperature and the moisture content of wheat on the number of eggs laid by these two insects will be discussed in another paper. In the experiments described in this paper the mortality of the immature stages of *C. oryzae* and *R. dominica* was determined at different temperatures in wheat of different moisture contents.

## EXPERIMENTAL TECHNIQUE.

Insects were obtained from laboratory cultures kept in wheat of 14 p.c. m.c. at 26° C. (*C. oryzae*) and 34° C. (*R. dominica*). A wheat of medium hardness (Seewari) was used in all experiments; its equilibrium moisture content at different relative humidities was determined, so that wheat of any desired moisture content could be obtained by desiccating over sulphuric acid of appropriate concentration.

The development of the insects from egg to adult was followed in single grains of wheat kept in jars of the type used for fruit preserving known as Fowler jars. The relative humidity of the air in the jars was controlled with sulphuric acid solutions.

*R. dominica.* A number of grains of known moisture content, each containing one insect of known age, were obtained by placing single eggs on grains of wheat and incubating each grain and egg in an enclosed cell of porous pottery[2] (cf. Birch, 1945). The eggs obtained as previously described (Birch, 1944) were not more than 12 hours old at 34° C. At all treatments they were incubated at 80 p.c. R.H. at the temperature of the experiment, for a few days, before being placed on the grains. By that time the cuticle had developed and the eggs could be separated without damage. The eggs were all incubated at 80 p.c. R.H. because at lower relative humidities the eggs did not retain their turgidity, and it was not found possible to separate them without damage. The mortality of eggs incubated for their full development at low humidities was determined previously (Birch, 1944); these figures were used in presenting the final table of mortality of the different stages. Of the eggs which hatched, the percentage of larvae which died without entering the grain and the percentage which died inside the grain were determined.

More insects survived in grain which had been damaged with a scalpel cut; for this reason experiments were carried out with both sound grain and grain which had been damaged in this way. A sufficient number of eggs was used in each treatment to ensure six replicates of 25 grains in which larvae had entered. At the low moisture levels where many insects died before entering the grain, hundreds of eggs were required for each treatment.

[1] The work was carried out with the aid of a research grant from the Federal grant to the University of Adelaide.

[2] A preliminary experiment showed that there was no significant difference in the survival when an egg was isolated with one or with 12 grains of wheat. The comparison was made at 38° C. in wheat of 14 p.c. m.c. and at 34° C. in wheat of 9 p.c. m.c. The method of using one grain of wheat, being the simpler, was therefore adopted.

TABLE 1.

*Showing mortality of different stages of* Rhizopertha dominica *at different temperatures in wheat of different moisture content in sound and damaged grain*.*

| Moisture content. | | 8. | | | 9. | | | 10. | | | 11. | | | 12. | | | 14. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stage. | | Egg. | 1st stage larva outside grain. | 1st stage larva inside grain. Total. | Egg. | 1st stage larva outside grain. | 1st stage larva inside grain. Total. | Egg. | 1st stage larva outside grain. | 1st stage larva inside grain. Total. | Egg. | 1st stage larva outside grain. | 1st stage larva inside grain. Total. | Egg. | 1st stage larva outside grain. | 1st stage larva inside grain. Total. | Egg. | 1st stage larva outside grain. | 1st stage larva inside grain. Total. |
| Temp. °C. | | | | | | | | | | | | | | | | | | | |
| 18·3 | Sound | | | | | | | | | | | | | | | | 8 | 41 | 49† *100* |
| | Damaged | | | | | | | | | | | | | | | | — | — | — — |
| 22·0 | Sound | | | | | | | 0 | 60 | 40 *100* | 0 | 54 | 35 *89* | 0 | 6 | 42 *48* | 0 | 13 | 40 *53* |
| | Damaged | | | | | | | 0 | 97 | *97* | — | — | — — | — | — | — — | — | — | — — |
| 26·0 | Sound | | | | 0 | 61 | 39 *100* | 0 | 18 | 67 *85* | 0 | 15 | 26 *41* | 0 | 3 | 30 *33* | 0 | 3 | 27 *30* |
| | Damaged | | | | 0 | 6 | 55 *61* | — | — | — — | — | — | — — | — | — | — — | — | — | — — |
| 30·0 | Sound | | | | 0 | 22 | 78 *100* | 0 | 7 | 67 *74* | 0 | 10 | 29 *39* | — | — | — — | 0 | 0 | 26 *26* |
| | Damaged | | | | | | | — | — | — — | — | — | — — | — | — | — — | 0 | 0 | 11 *11* |
| 34·0 | Sound | 30 | 70 | *100* | 20 | 26 | 52 *98* | 10 | 23 | 44 *77* | 0 | 4 | 37 *41* | 0 | 0 | 15 *15* | 0 | 0 | 22 *22* |
| | Damaged | 30 | 70 | *100* | 20 | 16 | 41 *77* | — | — | — — | — | — | — — | — | — | — — | — | — | — — |
| 36·0 | Sound | | | | 20 | 14 | 66 *100* | 10 | 27 | 51 *88* | 0 | 15 | 41 *56* | 0 | 4 | 35 *39* | 0 | 0 | 31 *31* |
| | Damaged | | | | 20 | 5 | 61 *86* | — | — | — — | — | — | — — | — | — | — — | — | — | — — |
| 38·2 | Sound | | | | | | | 55 | 16 | 29 *100* | 30 | 22 | 39 *91* | 25 | 18 | 33 *76* | 0 | 28 | 58 *86* |
| | Damaged | | | | | | | 55 | 37 | *92* | — | — | — — | 25 | 2 | 33 *60* | 0 | 10 | 13 *23* |
| 38·6 | Sound | | | | | | | | | | | | | | | | 0 | 100 | *100* |
| | Damaged | | | | | | | | | | | | | | | | 0 | 18 | 65 *83* |
| 39·0 | Sound | | | | | | | | | | | | | | | | 100 | — | — *100* |
| | Damaged | | | | | | | | | | | | | | | | | | |

Significant differences at P = 0·01. Egg = 17·0. First stage larva inside grain = 12·0. First stage larva outside grain = 18·5. Total mortality = 12·0. * Cf. text for definition of sound and damaged grain. † 2 p.c. reached final larval instar but died without pupating.

Experiments were carried out using sound grain at eight temperatures (four degree intervals between 18·2° C. and 34° C. and two degree intervals between 34° C. and 40° C.) and at the following moisture contents[3], 8 p.c., 9 p.c., 10 p.c., 11 p.c., 12 p.c. and 14 p.c. The experiments were repeated with damaged grain at ten combinations of temperature and moisture content of the wheat.

*C. oryzae*. Experiments with *C. oryzae* were more difficult; few eggs were laid at unfavourable temperatures or in grain drier than 11 p.c. m.c., and no technique was found adequate for providing large numbers of eggs of known age in wheat of low moisture content. It was not possible to use the method adopted with *R. dominica* as the eggs of *C. oryzae* are laid inside the grain. The procedure eventually adopted was to let the insects lay eggs in grain of 14 p.c. m.c. and then to dry the grain as rapidly as possible at the temperature and relative humidity of the experiment. Grains of 14 p.c. m.c. each containing one egg were placed on wire trays over sulphuric acid of the required concentration; the jars containing them were fitted with internal fans which maintained a circulation of air in the jar and accelerated the drying of the grain. The fan was operated for 8 hours each day until the moisture content of the wheat had come into equilibrium with the relative humidity of the atmosphere in the jar. The moisture content of control samples of wheat in the jar was determined at 10-day intervals. Eight replicates

[3] In accordance with the practice in the wheat trade moisture contents of wheat are expressed as a percentage of the original weight.

of 25 grains were used for each treatment. Experiments were carried out at 5 temperatures in wheat of the following p.c. moisture content, 9, 9·5, 10, 10·5, 11, 12 and 14. By the end of the tenth day the moisture content of the grain with within 0·5 units of the required value at each temperature except 18·2° C.; at 18·2° C. the moisture content of the grain was within one unit of the required value at the end of three weeks. The particular method of these experiments means that the figures obtained for mortality are on the low side.

## EXPERIMENTAL RESULTS.

The total mortality in wheat of 14 p.c. moisture content was 100 p.c. at temperatures of 18·2° C. or lower and 39° C. or higher. Some insects completed their development in wheat of 9 p.c. m.c. (at 34° C.) but none survived in wheat of 8 p.c. m.c. (Table 1).

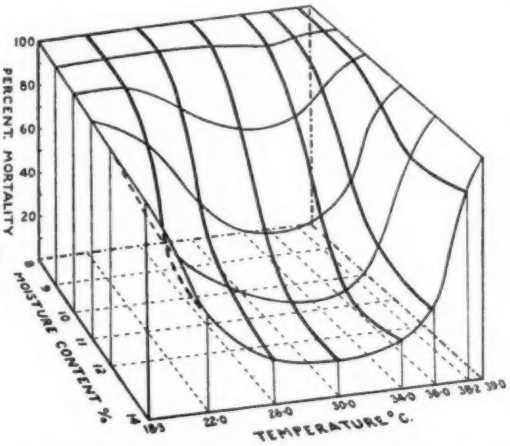


**Fig. 1.** A three-dimensional graph showing the influence of temperature and the moisture content of wheat on the mortality of *Rhizopertha dominica* in its development from the egg to adult stage.

The influence of temperature and the moisture content of wheat on the mortality of *R. dominica* is illustrated in the three-dimensional graph, Fig. 1. The height of the vertical co-ordinate at any point represents the mortality. The section of the diagram in which the lines lie on the top surface of the cube shows the combinations of temperature and moisture content which give 100 p.c. mortality. The vertical sigmoid curves show the trend of mortality plotted against moisture content at each temperature; these curves resemble dosage mortality curves, although the points do not fall on a straight line when plotted as probits on a plane surface. The three sigmoid curves for 26° C., 30° C. and 34° C. all lie on about the same plane surface; this is because there is no significant difference in mortality at any one moisture content at these three temperatures (excepting that between 34° C. and 26° C. at 12 p.c. m.c.). There was no significant difference in mortality at 14 p.c. and 12 p.c. m.c. at any temperature. As the temperature was increased beyond 34° C. or reduced below 26° C. mortality was significantly greater at any chosen moisture content. This is shown in the U-shaped horizontal curves in Fig. 1. The flattening out of the U at low moisture contents is due to the increased mortality at all temperatures at these moisture contents. There was a statistical interaction between temperature and the moisture content of grain, i.e. a particular reduction in moisture content resulted in different percentage increases in mortality at different temperatures. The lowest moisture content at which there was any survival varied with temperature; it became lower as the temperature increased from 18·2° C. to 34° C. and became higher as the temperature rose above 34° C.

More than 98 p.c. of the mortality of post-embryonic stages occurred in the first larval stage (Table 1). After emerging from the egg the first stage larva is exposed to the air until such time as it succeeds in penetrating the grain. When the period of exposure is long or when the environment is severe, the mortality rate of the first stage larva and therefore too the total mortality would be expected to be higher. The activity of the first stage larva may be greatest at 34° C. (the rate of development of the larva is at its maximum at this temperature), the period during which the larva would be exposed to the air would then be shortest at 34° C.; this may explain why the mortality rate was lowest at 34° C. and why there was some survival of insects in grain of 9 p.c. at 34° C., but at no other temperature.

When the grains of wheat were damaged with deep scalpel cuts there was a higher survival of insects and some insects survived in damaged grain at temperatures and moisture contents at which there was no survival in undamaged grain. This was due to the greater number of first stage larvae which succeeded in entering damaged grain and becoming established. In both damaged and undamaged grain the number of insects which died in stages other than the first instar larva was so small that separate categories are not shown in the Table.

In "sound" grain, that is grain that is sound in the sense of not possessing any cracks in the endosperm extending to the surface, the first stage larva generally entered the grain at the embryo end; at this end the testa is often lacerated or loose. At 30° C. and 34° C., 80 p.c. of 400 first stage larvae entered grain of 10 p.c. m.c. at the embryo end, the rest entering at the middle or end of the crease. In damaged grain the larvae always entered by the damaged surface.

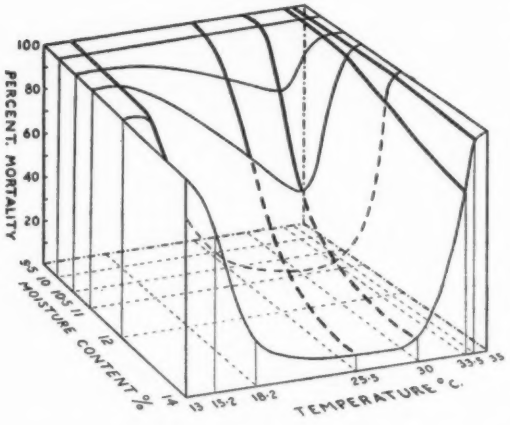


**Fig. 2.** A three-dimensional graph showing the influence of temperature and the moisture content of wheat on the mortality of the small strain of *Calandra oryzae* in its development from the egg to adult stage.

*C. oryzae.* The driest grain in which there was any survival of *C. oryzae* contained 10·5 p.c. moisture; even at 11 p.c. m.c. mortality was high (Table 2). There was 100 p.c. mortality in wheat of 14 p.c. m.c. at temperatures less than 15° C. and at temperatures higher than 34° C.

The influence of temperature and the moisture content of the wheat on mortality is illustrated in Fig. 2; owing to the absence of figures for mortality in wheat of 12 p.c. m.c. at 25·5° C. and 30° C. the diagram is not complete, but hypothetical lines have been dotted in through these points. The scale is not the same as that in Fig. 1, but it can be seen that the general form of the figure is much the same as that for *R. dominica.* The trough in the figure is narrowly V-shaped rather than U-shaped; this is due to the fact that the mortality was a minimum at 30° C. at all moisture contents, and it was greater at temperatures higher or lower than 30° C. at all moisture contents except 12 p.c. m.c. For *R. dominica*, on the other hand, there was a zone of 8° C. (26° C.–34° C.) in which there was no significant difference in mortality. As with *R. dominica* there was a statistical interaction between temperature and moisture content, and the moisture content of the driest grain in which there was any survival was higher as temperature increased above 30° C. or decreased below 18·2° C.

TABLE 2.

*Showing the mortality of* C. oryzae *in its development from egg to adult in grain of different moisture contents at different temperatures.*

| | Moisture content of wheat. | | | | |
|---|---|---|---|---|---|
| Temp. °C. | 10. | 10·5. | 11. | 12. | 14. |
| 13·0 | | | | | 100 |
| 15·2 | | | | 100 | 75 |
| 18·2 | 100 | 99 | 86 | 39 | 12 |
| 25·5 | 100 | 88 | 64 | — | 7 |
| 30·0 | 100 | 76 | 38 | — | 10 |
| 33·5 | | 100 | 97 | 88 | 75 |
| 34·0 | | | | 100 | 97 |
| 35·0 | | | | | 100 |

At P = 0·01 a difference of 10 is significant.

At all combinations of temperature and moisture content shown in the Table the mortality of the egg stage was uniformly low except at 13° C. and 34° C.; it has therefore not been shown separately. In grain of 9 p.c., 9·5 p.c. and 10 p.c. moisture content the total mortality of post-embryonic stages occurred in the first stage larva. In wheat of higher moisture content most of the mortality occurred in the first stage larva (over 90 p.c.); the mortality at later stages was probably due to the fact that the wheat became drier during the course of the experiment (see page 142).

## SUMMARY.

*Calandra oryzae* (small strain) and *Rhizopertha dominica* have been reared from egg to adult at different temperatures in wheat having different moisture contents. The mortality in the immature stages was recorded.

The maximum temperatures at which there was any survival were 38·6° C. for *R. dominica* and 34° C. for *C. oryzae*. The minimum temperatures were 22° C. and 15·2° C. and the driest grain in which there was any survival was 9 p.c. and 10·5 p.c. respectively.

The relation between temperature, moisture content of grain and mortality has been represented on three-dimensional graphs. For both insects there was an interaction of temperature and moisture content in their influence on mortality.

The mortality of *R. dominica* was uniformly low in grain of 12 p.c. and 14 p.c. moisture content at temperatures between 26° and 34° C. At temperatures outside this range and in drier wheat mortality was significantly greater.

The driest wheat in which *R. dominica* developed contained 9 p.c. moisture; the mortality at 9 p.c. was over 95 p.c. in sound grain and over 60 p.c. in damaged grain.

The moisture content of the driest grain in which *R. dominica* was able to develop varied with the temperature, falling as the temperature increased from 18·2° C. to 34° C. and rising as the temperature increased from 34° C. onwards.

There was a higher survival of *R. dominica* in grain which had been damaged; this was due to the greater facility with which first stage larvae were able to enter the grain and become established. The mortality of *C. oryzae* increased rapidly as the moisture content fell below 14 p.c. The mortality was lowest at 30° C. at all moisture contents. At temperatures greater or less than 30° C. mortality was greater. The moisture content of the driest grain in which there was any survival of *C. oryzae* was higher as the temperature increased above 30° C. or decreased below 18·2° C.

Practically all the mortality of immature stages occurred in the first stage larva. In wheat of 14 p.c. m.c., which is the most favourable level of moisture for both insects, the mortality of *R. dominica* was higher than that of *C. oryzae* within their respective medial ranges of temperature. But the difference between the mortality of *R. dominica* at 14 p.c. m.c. and lower moisture contents was less than the corresponding difference for *C. oryzae*.


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# THE SIGNIFICANCE OF PRIMARY ISOLATION OF INFLUENZA VIRUS BY INOCULATION OF MICE OR OF THE ALLANTOIC CAVITY OF CHICK EMBRYOS

by F. M. BURNET AND J. D. STONE[1]

(From the Walter & Eliza Hall Institute, Melbourne).

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In recent papers from this laboratory (Burnet and Bull, 1943; Beveridge and Williams, 1944) it has been stated that influenza virus A as found in the throat washings from a typical human case differs sharply from standard laboratory strains but can readily be transformed to take on the standard character. The virus in its normal form as a human pathogen was said to exist in the O phase differentiated from the D phase, characteristic of laboratory strains propagated in mice or the allantoic cavity of chick embryos, in the following respects.

(1) O phase virus fails to agglutinate fowl erythrocytes although it is active against human and guinea pig cells.

(2) It is unable to multiply in the allantoic cavity. Active growth takes place in the amniotic cavity or in the yolk sac.

(3) O phase virus has little or no power to infect mice as judged by subsequent development of immunity. Ferrets are readily infected with clinical symptoms and guinea-pigs subclinically.

(4) Haemagglutination by O phase virus is much less readily inhibited by human tears than the agglutination produced by well adapted D strains.

These findings are at variance with the reports that have appeared in regard to (1) the isolation of virus directly in mice in the sense either of developing a pathogenic strain by passage (Francis and Magill, 1937) or of recognizing the subsequent acquisition of immunity to a virulent strain as a result of inoculation the suspected material (Burnet and Foley, 1941). (2) The isolation of virus by allantoic inoculation and passage (Thigpen and Crowley, 1943; Adams, Thigpen and Rickard, 1944).

As it seemed unlikely that in other times and places human influenza should be due to D phase virus we have studied the behaviour of O virus in the allantoic cavity and in mice in an attempt to resolve the apparent discrepancies.

The strain used was BEL in its 25th to 30th passage in the O phase and all the experiments were initiated with embryo lung emulsions initially of O type and further "purified" by two absorptions with fowl red cells according to the technique described previously (Burnet and Stone, 1945).

*Multiplication of O Phase Virus in Mouse Lung.*

The most complete experiment of this series is summarized in Table 1. The initial O virus after absorption with fowl cells had a titre of F/G 0/480. It was titrated amniotically and in the allantoic cavity as well as being inoculated at each dilution into 8 mice. Two mice of each group were killed at 2 days and a pooled lung emulsion titrated amniotically for its content of O and D virus. The remaining 6 were tested for immunity two weeks later with a mouse-virulent strain of influenza virus A (Melbourne).

[1] This work was carried out under a grant from the National Health and Medical Research Council for work on virus diseases.

TABLE 1.

*Behaviour of O virus in mice.*

| | Chick embryo titration. | | | Intranasal inoculation in mice. Amniotic titration of lung. | | | | |
|---|---|---|---|---|---|---|---|---|
| Dilution of O inoculum. | Amniotic. | Allantoic. | Development of immunity. | Undiluted. | $10^{-1}$. | $10^{-2}$. | $10^{-3}$. | $10^{-4}$. |
| $10^{-3}$ | OO– – | | 0/6 | | | | | |
| $10^{-5}$ | OOω̃ω̃ | 0/4 | 0/6 | — | . . . | — | | |
| $10^{-4}$ | OOω̃δ | 2/4 | 2/6 | — | . . . . | — | | |
| $10^{-3}$ | ω̃ω̃δδ | 4/4 | 4/6 | — | . . . . | — | | |
| $10^{-2}$ | | 4/4 | 6/6 | . . . | DDDδ | OOω̃δ | OOOω̃ | – – – – |
| $10^{-1}$ | | 4/4 | 6/6 | | | | | |

O, ω̃, δ, D: virus in amniotic fluid giving agglutination of type indicated; –, no agglutination.

Allantoic results indicate number of positive fluids over number of embryos inoculated.

Immunity in mice is shown by the number surviving a challenge inoculation of "Melbourne" virus (1: 100 allantoic fluid) which killed all control animals.

Table 1 shows the results obtained from what was essentially a titration of O virus by four different methods. It will be seen that to a first approximation the development of δ or (D) virus after amniotic inoculation corresponded (a) to power to multiply in the allantoic cavity and (b) power to immunize mice. With dilutions $10^{-5}$ and $10^{-6}$ in which only O virus was initially present the only evidence for the existence of virus was obtained from the amniotic inoculations. The results of amniotic titration of lung emulsions from the mice are, however, not in accord with the simple view that δ or D phase virus can multiply in mice but not O or ω̃. The only positive result was obtained from mice which received approximately $10^4$ infective doses of O virus and 10–100 of δ virus. From this inoculum virus had developed in 2 days which still showed about 100 times as much O or ω̃ virus as δ or D, in other words O virus had multiplied about as freely as D in one or both of the mice providing this material. In another experiment along similar lines the only O virus obtained from mouse lung was from mice inoculated with $10^{-4}$ dilution of the original O inoculum. The mice receiving virus diluted $10^{-2}$ gave only D type material at 2 days.

Extensive experiments on the immunizing power of O and D virus for mice were described in our first communication of the O–D phenomenon. These showed that the amount of virus required to immunize mice against a challenge dose of virulent (D) virus was much smaller in the D phase than in the O phase. The overall results suggested that the whole of the immunizing capacity may have been due to the D virus unavoidably present in all the O material. Taking these into account with the current experiments it appears that there is no absolute inability of O virus to multiply in the respiratory tract of the mouse. The probability of a given virus particle in the O phase initiating infection in this situation is, however, very much less than for a D phase particle.

*Multiplication of O Virus in the Allantoic Cavity.*

It has been noted in previous papers (Burnet and Bull, 1943; Burnet and Stone, 1945) that on occasion one obtains from a series of embryos inoculated allantoically with serial dilutions of O virus a few fluids that give F/G ratios indicating O or ω̃ type virus. These are of low titre, G being 50 or less but they appear to be genuine O phase virus since amniotic titration of the fluids give O type results at limiting dilutions.

In a further study of the capacity of O virus to multiply in the allantoic cavity tests for the presence of O virus were made by subinoculation into the amniotic cavity as well as by direct tests with guinea-pig cells.

The experiment whose results are detailed in Table 2 gives a clear indication of the position. The usual parallel titrations of an absorbed O virus (embryo lung emulsion) were made by amniotic and allantoic inoculation. Amniotic infections were obtained to a dilution of $10^{-3}$ but in the allantois dilutions $10^{-4}$, $10^{-5}$, $10^{-6}$, were all negative by haemagglutination tests. At $10^{-3}$ one δ fluid was obtained and one ω̃ (F/G 4/48) with two negative embryos. The negative fluids from the embryos of the $10^{-4}$, $10^{-5}$, $10^{-6}$ series were pooled for each dilution and subinoculated both into the allantoic cavity (groups $B_4$, $B_5$, $B_6$ in the Table) and into the amniotic cavity. A third allantoic passage was made in the same fashion.

TABLE 2.

*Behaviour of O virus in allantoic cavity of chick embryo.*

| Dilution of O inoculum. | Chick embryo titration. Amniotic. | Allantoic. | Second passage. Amniotic. | Allantoic. | Third Passage. Amniotic. | Allantoic. | Fourth passage. Amniotic. | Allantoic. |
|---|---|---|---|---|---|---|---|---|
| $10^{-7}$ | OO – x | | | | | | | |
| $10^{-6}$ | O – – x | 0/4 (A6) | – – – | 0/3 (B6) | – – – – | 0/4 (C6) | – – – – | 0/4 |
| $10^{-5}$ | OOO$\tilde{\omega}$ | 0/4 (A5) | OO – – | 0/3 (B5) | – – – x | 0/4 (C5) | – – – – | 0/4 |
| $10^{-4}$ | | 0/4 (A4) | OOO | 0/3 (B4) | DDDx | 4/4 | | |
| $10^{-3}$ | | $\tilde{\omega}\delta$ – – | | | | | | |
| $10^{-2}$ | | $\tilde{\omega}\delta\delta$ – | | | | | | |

Two further passages by allantoic inoculation are shown from those allantoic fluids (A4, A5, A6) which were obtained from embryos inoculated with O virus but gave no direct evidence of virus. Each passage is tested in parallel by amniotic inoculation.

Symbols as in Table 1. x = nonspecific death of embryo.

If our working hypothesis is correct that influenza virus in its normal condition as a human pathogen is in the O phase then the series of allantoic inoculations described is strictly analogous to the procedure used for primary isolation by Thigpen and Crowley. The only difference is that instead of using primary throat washings of unknown virus titre we have started with three inocula containing known concentrations of O virus in the ratio 1: 10: 100. If the results were reported in the form used by Rickard the two weaker preparations would be given as negative after 3 passages, the strongest as positive at the 3rd passage. The associated amniotic inoculations indicate in addition what has occurred in the "negative" allantoic passages. The $10^{-5}$ inoculum has shown a minimal degree of multiplication probably in only one of the four embryos used and the next allantoic inoculation has resulted in the disappearance of the virus. The largest inoculum $10^{-4}$ has initiated definite multiplication in the allantois although none of the fluids showed any trace of direct haemagglutinating capacity even when a small amount of guinea-pig cells was allowed to sediment through 4 or 5 ml. of undiluted fluid. Even in the next passage, however, none of the allantoic fluids showed manifest agglutinating power. One showed a trace of agglutination which was unfortunately obscured by accidental haemorrhage into the fluid and from the results with the next passage it is highly probable that if the embryos (B4 in the Table) had been incubated for 1 or 2 more days they would have given positive haemagglutination.

## DISCUSSION.

No direct confirmation of the phenomenon which we have described as O–D mutation in influenza virus A has yet been reported from any other laboratory.[2] Nevertheless there is very little to be found in the published accounts of primary isolation of the virus that conflicts with the view that natural human infection by influenza virus A is *always* due to the O phase.

The well known fact that it is difficult to establish virus in mice until several ferret passages have been made speaks distinctly for this interpretation. The fact that infection can occasionally be initiated by intranasal inoculation in mice or by allantoic inoculation in chick embryos does not necessarily indicate that the human virus concerned was not in the O form. The present work makes it much more likely that there is no categorical distinction between O and D virus in regard to limitations of infection and growth. There is a certain probability that O virus will initiate infection in mouse lung or allantois but this probability is far smaller than the corresponding one for ferret or amniotic cavity.

Our observations on the O–D character have had to be made on strains isolated by ourselves and any generalizations from these experiments will only be valid if the strains isolated in Australia in 1942–43 conform to the general charac-

[2] Partial confirmation of our findings is to be found in papers by Eaton *et al.* (Proc. Soc. exp. Biol. and Med., 58, 6, 1945) and Hirst (*ibid.*, 58, 155, 1945) received since this paper was submitted for publication.

ter of those isolated in other parts of the world in recent years. With this proviso the following brief notes are given to summarize the significance of the various methods for the primary isolation of influenza virus A.

*Ferret inoculation.* O virus multiplies readily in the respiratory tract. There is insufficient evidence to state whether the D phase eventually overgrows the O with repeated passage from ferret to ferret. The increase in actual or potential mouse pathogenicity with ferret passage suggests that it does.

*Amniotic inoculation in embryos of 13 to 15 days' incubation.* O virus multiplies readily and invades the embryo lung which becomes the most satisfactory source of virus for continued passage in the O phase. Embryos of 12 days or younger are much less satisfactory giving a smaller proportion of infections and a greater proportion of D phase fluids.

*Yolk sac inoculation in 6 day embryos.* Multiplication occurs in the O phase but the method is not so sensitive as amniotic inoculation and provides less convenient material. It is not suitable for attempts at isolation from non-sterile material treated with a bacteriostatic agent.

*Allantoic inoculation in 11-day embryos.* O virus will fail to initiate infection except when in relatively large amount ($10^2$–$10^4$ amniotic infective doses). Only a proportion of the embryos in which infection has been initiated will show visible haemagglutination after 3 days' incubation. Subinoculation from apparently negative embryos will increase the proportion of positive results but the method is far less sensitive than the amniotic technique.

*Intranasal inoculation in mice.* Here the position is very similar to that found with allantoic inoculation. Large inocula of O virus will irregularly initiate infection and give rise to demonstrable immunity. Continued passage would very rarely allow the development of the D phase and eventual mouse virulence.

## SUMMARY.

O phase influenza A virus will, when in large enough concentration, initiate infection irregularly in mouse lung or in the allantoic cavity of the chick embryo.

The amniotic cavity of the chick embryo of 13–15 days' incubation is susceptible to infection by much smaller amounts of virus and is correspondingly to be preferred as a medium for primary isolation.

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# FURTHER STUDIES ON THE O-D CHANGE IN INFLUENZA VIRUS A

by F. M. BURNET AND J. D. STONE[1]

(From the Walter & Eliza Hall Institute, Melbourne).

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In a previous paper (Burnet and Bull, 1943) it was shown that influenza A virus when isolated directly from the human throat in the amniotic cavity of the chick embryo differs in two characteristics from stock strains of influenza virus. It agglutinates fowl cells weakly or not at all while agglutinating guinea-pig cells to a normal titre and it fails to multiply when placed in the allantoic cavity. Virus in this "O phase" rapidly changes to the "D phase" on passage, thereby taking on the familiar power to agglutinate fowl cells and to grow in the allantoic cavity. It can, however, usually be maintained in the O phase by passage at limiting dilutions in the amniotic cavity or in the yolk sac.

The primary reason for undertaking further work on the O–D phenomenon was the need to have available virus in the O form for studies on the possibility of developing methods of isolation without filtration. This work will be reported separately. The present communication is essentially an account of experiments to find the best method of maintaining influenza A in the O phase through repeated passage and of ensuring that when required, O virus can be produced with regularity.

## MATERIALS AND TECHNIQUE.

In all the experiments to be described the strain BEL isolated in 1942 was used. This is an influenza A virus which in the adapted D phase is serologically very close to "Melbourne" or PR8. It had been stored as 11th passage amniotic fluid in a solid $CO_2$ refrigerator for 20 months. After thawing the fluid gave almost the same haemagglutination reactions as it showed in 1942, a fowl cell titre of 1: 5 and a guinea-pig cell titre of 1: 160 (F/G titre 5/160). Amniotic titrations in 11 day embryos indicated a fall in infective titre but one embryo inoculated at a dilution of 1: $10^4$ gave a typical O fluid (92164). Titrations of this fluid by the amniotic route showed the typical behaviour, O virus being obtained with the highest dilutions while fluids of D or intermediate characteristics were obtained from lower dilutions.

The methods of amniotic inoculation, haemagglutination tests and allantoic titration were essentially the same as previously used.

Emulsions were prepared from embryo lungs by washing once with saline, grinding with quartz powder and adding 1 ml. of saline to each pair of lungs. After further emulsification by repeated suction and expulsion through a capillary pipette the suspension was centrifuged (or if more convenient allowed to settle overnight in the refrigerator) and the supernatant fluid used for haemagglutination or subinoculation.

### A. *The Types of Red Cell Agglutination Shown by Virus at Various Stages of Adaptation.*

In previous work we considered that a strain of virus was in the O form if the titre shown against fowl cells was one-third or less of that given with guinea-pig or human cells. Further experience has shown that an almost continuous series of reactions can be observed amongst amniotic fluids or lung emulsions from embryos inoculated with O type virus at various dilutions. We have now adopted the practice of confining the term "O phase" to preparations which show no fowl cell agglutination or at most a titre less than 1/20 of that shown with guinea-pig cells.

With our present technique we have had no difficulty in maintaining the strain BEL in such a state that limiting dilutions inoculated amniotically give amniotic fluid or lung emulsions which show no fowl cell agglutination whatever at dilutions of 1: 5 or higher despite guinea-pig titres which range to 1: 640. Without exception any inoculum which when inoculated amniotically

[1] This work was carried out under a grant from the National Health and Medical Research Council.

gives rise to fluids or lung tissues with this "complete" O reaction, fails to multiply in the allantoic cavity.

Many preparations give agglutination reactions which are intermediate between those of such O phase preparations and those of a typical D fluid with fowl cell titre equal to or higher than the guinea-pig cell titre. Most of the amniotic fluids described in earlier work as O would fall into the category which we now refer to as $\tilde{\omega}$. These show some agglutination of fowl red cells to a titre 1/20 to 1/4 of the guinea-pig cell titre. The fowl cell agglutination takes the form of rather coarse loose aggregates of cells which rapidly slide to the bottom of the tube leaving a central accumulation of cells with only a fringe of rather pale-looking cell aggregates at the edge. If left to stand beyond an hour or two all evidence of agglutination is liable to disappear. This is the general type of reaction which we described for O strains previously and like the full O reaction it is normally given only by material which fails to grow in the allantoic cavity. One exception to this statement is described in a later section.

The second intermediate type $\delta$ gives fowl cell agglutination of character intermediate between $\tilde{\omega}$ and typical D. The agglutination is more compact than $\tilde{\omega}$, the aggregates are denser and deeper in colour and they adhere more definitely to the glass although never forming a continuous coating. When the tubes are read after standing about 75 minutes at room temperature the cell aggregates have mostly accumulated at the bottom of the tube but some still adhere to the sides giving appearances which we have called streaky (with radially distributed residues along the tracks taken by aggregates slipping to the bottom) or insular (with fairly large clumps of cells held up at various points on the hemispherical surface of the bottom of the tube). The F/G titres show ratios from 1/3 to 1/1 and virus which gives this type of agglutination after amniotic inoculation will multiply in the allantoic cavity.

Our criterion for true D type agglutination is that at intermediate dilutions a complete "shield" of finely aggregated cells should adhere to the glass for a time at least, slipping when it occurs taking the form of relatively large "landslides" which give a characteristically ragged appearance to the deposit.

The range from O through $\tilde{\omega}$ and $\delta$ to D obviously corresponds to an increasing efficiency of the bridging process by which the virus can hold fowl red cells together. Both the increasing value of the F/G ratio and the changing pattern of the agglutinated and deposited cells speak plainly for this.

TABLE 1.

*Types of fowl cell agglutination.*

| Type. | Character of agglutination. | F/G ratio. | Multiplication in allantoic cavity. |
|---|---|---|---|
| O | No agglutination | 0/40–0/1,000 | Nil |
| $\tilde{\omega}$ | Weak loose agglutination rapidly disappearing | 1/20–1/3 | Nil |
| $\delta$ | Streaky or insular agglutination | 1/3 –1 | + |
| D | Continuous shield often with ragged "landslides" | 1 or + | + |

The type of agglutination is only slightly altered by the physical character of amniotic fluid. If amniotic fluid and lung emulsion from the same embryo and of approximately equal guinea-pig cell titre are tested against fowl cells, the intermediate types of agglutination $\tilde{\omega}$ or $\delta$ will usually be similar for both sorts of virus. With amniotic fluid virus the lower dilutions tend to give aggregates slipping more readily toward the bottom of the tube.

Slight differences in the character of agglutination may be seen when the same virus is tested against cells from several individual fowls. In some instances borderline types of virus would be placed in different categories according to whether more or less sensitive cells were used. All the differences between red cells can, however, be referred to a simple range of agglutinability. With more agglutinable cells the points at which a continuous range of virus samples is divided into the four categories are each moved slightly toward the O end of the scale as compared with the division given with less agglutinable cells. A virus O by one set of cells may show a trace of $\tilde{\omega}$ type agglutination with a more agglutinable cell suspension but will never show agglutination approaching $\delta$ or D character.

In a similar way the results differ slightly when agglutination takes place at different temperature. In the cold the agglutination is more complete than at room temperature, i.e. an intermediate tends toward the D character.

The distribution of the types of virus can be best demonstrated from the results of a comparative amniotic-allantoic titration of a typical O amniotic fluid. The following detailed titration passage (18th O passage BEL) gives a typical enough picture of the findings. Amniotic fluid BEL 93911 was serially diluted in cold horse serum saline (10 p.c.) and inoculated amniotically and into the allantoic cavity.

TABLE 2.

*Comparative amniotic-allantoic titration of an O virus.*

| Dilution. | Amniotic. | | Allantoic. |
|---|---|---|---|
| $10^{-7}$ | AF | --- | |
| | L | --- | |
| $10^{-6}$ | AF | OOOOO$\tilde{\omega}$ | ---- |
| | L | OOOOO $\tilde{\omega}$ | |
| $10^{-5}$ | AF | $\tilde{\omega}\tilde{\omega}\tilde{\omega}\tilde{\omega}$ | ---- |
| | L | $\tilde{\omega}\tilde{\omega}\tilde{\omega}\tilde{\omega}$ | |
| $10^{-4}$ | AF | $\tilde{\omega}\delta$D | $\delta\delta\delta$D |
| | L | $\delta\delta\delta$ | |
| $10^{-3}$ | AF | $\delta\delta\delta\delta$ | DDDD |
| | L | --$\delta\delta$ | |
| $10^{-2}$ | AF | $\delta\delta\delta\delta$ | DDDD |
| | L | ---$\delta$ | |
| $10^{-1}$ | AF | $\tilde{\omega}$*$\delta$*$\delta\delta$ | |
| | L | $\delta$*$\delta$*-- | |

AF, Amniotic fluid. L, Lungs emulsified in 1 ml. saline. O, $\tilde{\omega}$, $\delta$, D, Types of red cell agglutination described in text. *, These embryos were examined after 2 days, all others after three days. –, No agglutination of guinea-pig cells.

Except for 2 eggs inoculated with 1: 10 virus which were opened on the second day, all the amniotic eggs were examined 3 days after inoculation, F/G titrations being made of amniotic fluid and of an emulsion prepared by grinding the washed embryo lungs in 1 ml. of saline. As examples of the detailed findings the results of amniotic inoculations of virus 1: 10 and 1: $10^6$ were.

$10^{-1}$ Eggs opened at 2 days AF 40/120, L 280/360; AF 160/500, L 200/320.
Eggs opened at 3 days AF 320/640, L 0/0; AF 240/320, L 0/0.
$10^{-6}$ AF 0/160 0/40 0/120 0/70 0/160 280/480.
L 10/320 0/10 0/640 0/480 0/300 100/120.

The allantoic readings were made at 3 days, detailed F/G titrations were not done, the diagnosis of type being made from the appearance of the agglutination of fowl and guinea-pig cells by a single dilution (1: 5) of each fluid.

Points to be noted are (1) the change in character as lower dilutions are approached, (2) the correspondence in type between lung and amniotic fluid, (3) the disappearance of virus from the lung between 2 and 3 days when the inoculum was a heavy one, (4) infection of the allantoic cavity results only when inocula giving $\delta$ or D infections amniotically were used.

When similar titrations are made with virus showing $\tilde{\omega}$ agglutination the results differ significantly from O but seem to fall into two different categories. Two examples typifying these categories are shown in Table 3.

TABLE 3.

*Comparative amniotic-allantoic titration of $\tilde{\omega}$ viruses.*

| AF 93996 40/160 $\tilde{\omega}$. | Amniotic. | Allantoic. | AF 94509 25/240 $\tilde{\omega}$. | Amniotic. | Allantoic. |
|---|---|---|---|---|---|
| $10^{-1}$ | O $\tilde{\omega}\tilde{\omega}$ | ---- | $10^{-1}$ | $\tilde{\omega}\tilde{\omega}\tilde{\omega}\tilde{\omega}$- | ++++ |
| $10^{-4}$ | $\tilde{\omega}\delta$DD | ++-- | $10^{-4}$ | $\tilde{\omega}\tilde{\omega}$++++ | ++++ |
| $10^{-6}$ | $\delta$DD | +++- | $10^{-6}$ | $\delta\delta$+ | ++++ |

\+ Agglutination of guinea-pig cells but type not determined.

In the first example O and $\tilde{\omega}$ fluids are obtained from amniotic inoculation at limiting dilutions and not from the corresponding allantoic inoculations. The most likely interpretation of this type of reaction is that it represents a mixture containing a slight excess of O with $\delta$ (or D) virus. An almost precise imitation of this behaviour is shown by an artificial mixture of this sort (see Table 4).

The second type, e.g. 94509, differs in that $\tilde{\omega}$ type reactions are produced in the amnion by dilutions which also infect the allantoic cavity. Though they were not tested in this case the allantoic fluids from the higher dilutions give an $\tilde{\omega}$ type reaction.

An exceptional example of this type was encountered in an allantoic fluid infected at limiting dilution by an O lung emulsion. This fluid gave an F/G titre of 0/20. When titrated amniotically it was active to $10^{-7}$ giving at that dilution fluids some of which showed only a trace of fowl agglutination.

TABLE 4.

*Comparative titration of an aberrant allantoic fluid.*

| Dilution. | Amniotic. | Allantoic. |
|---|---|---|
| $10^{-7}$ | OO $\tilde{\omega}\tilde{\omega}\tilde{\omega}$ | ------ |
| $10^{-6}$ | O $\tilde{\omega}\tilde{\omega}$ -- | ------ |
| $10^{-5}$ | $\tilde{\omega}\tilde{\omega}\tilde{\omega}\tilde{\omega}\tilde{\omega}\delta$ | $\tilde{\omega}\tilde{\omega}$OO$\delta$ --- |
| $10^{-4}$ | | O$\tilde{\omega}$DD |
| $10^{-3}$ | | DDDD |

O indicates only a trace of fowl cell agglutination, in the case of the allantoic fluids associated with a low guinea-pig cell agglutination.

B. *Comparison of Results of Amniotic Inoculation in Embryos of Different Ages.*

Most of our previous work was done with embryos inoculated amniotically at 11–12 days incubation. In view of the suggestion from several experiments that multiplication took place mainly in the lung epithelium, tests were made to see whether growth occurred more readily in older eggs.

A virus (92164 amniotic fluid F/G 0/70) was known to produce O infections when inoculated at a dilution of 1 : $10^6$ and this dilution was inoculated amniotically into 20 embryos of each age, 11, 12 and 13 days. After 2 days' incubation a few embryos in each series were tested by the withdrawal of a little amniotic fluid, the eggs being then resealed and returned to the incubator. Further tests were made at 3 days; most of the 13-day eggs were opened at this time while all the remainder were finally examined at 5 days. F/G titrations of all positive fluids were made.

TABLE 5.

*Typical results with 11, 12 and 13 day embryos inoculated with O virus diluted $10^{-6}$.*

| 11 day embryos. | | | 12 day embryos. | | | 13 days embryos. | |
|---|---|---|---|---|---|---|---|
| 2 days. | 3 days. | 5 days. | 2 days. | 3 days. | 5 days. | 2 days. | 3 days. |
| — | — | — | — | — | †— | 0/30 | 0/120 |
| — | — | — | | — | 40/160 $\tilde{\omega}$ | 40/220 $\tilde{\omega}$ | 80/280 $\tilde{\omega}$ |
| — | — | — | | — | 0/25 | 0/5 | 10/160 |
| — | — | 120/160$\delta$ | | — | †0/8 | 0/120 | 0/360 |

Results of F/G titrations of amniotic fluids are shown.

— Indicates that a sample of amniotic fluid taken on the day shown gave no agglutination of guinea-pig cells. Each horizontal group of results pertains to a single embryo. See text for fuller description.

TABLE 6.

*Summary of results with embryos of different ages.*

| 11 day embryos. | | | 12 day embryos. | | | 13 day embryos. | | |
|---|---|---|---|---|---|---|---|---|
| 2 days. | 3 days. | 5 days. | 2 days. | 3 days. | 5 days. | 2 days. | 3 days. | 5 days. |
| 4 — | 8 — | 2 0 | 6 — | 1 0 | 5 0 | 4 0 | 10 0 | 4 0 |
| 6 + | 2 + | 1 $\tilde{\omega}$ | 2 + | 6 — | 2 $\tilde{\omega}$ | 1 $\tilde{\omega}$ | 1 $\tilde{\omega}$ | 1 — |
| | | 2 $\delta$ | | 5 + | 2 $\delta$ | 1 — | 1 — | |
| | | 6 — | | | 1 D | 1 + | 1 + | |
| | | 1 + | | | 1 — | | | |
| | | | | | 2 + | | | |
| | 5/12 + | | | 10/13 + | | | 16/18 + | |

The total shows the proportion of embryos surviving more than 3 days which showed evidence of infection.

It will be seen that there is a striking advantage in using the older embryos both in regard to the proportion showing evidence of infection and in the much higher proportion of O type fluids obtained. In most subsequent work 13-day embryos have been used.

Still older embryos were included in a later experiment in which the inoculum was an O type lung emulsion which had been absorbed with fowl cells (see later). This produced O infections over the range of dilutions from $10^{-4}$ to $10^{-7}$. Embryos which had been incubated at 99·5° F. for 12, 14, 15, 16 and 17 days were inoculated into the amnion with dilutions of $10^{-5}$ and $10^{-6}$. About half the eggs were tested by withdrawal of amniotic fluid after two days' further incubation and some of those positive at that stage were opened for examination of the lungs. All the remainder were finally opened on the third day and F/G titrations made of each lung emulsion. Table 7 contains the information derived from this experiment.

TABLE 7.

*Result of amniotic inoculation of dilute O virus (95306 absorbed with fowl cells) in embryos of ages from 12 to 17 days.*

| Age of embryos at inoculation. | Dilution $10^{-5}$. At 2 days. | Dilution $10^{-5}$. At 3 days. | Dilution $10^{-6}$. At 3 days. |
|---|---|---|---|
| 12 days | – – – – | – – – – | – – |
| 14 days | OO – | OO ω̃ω̃ | OOOO |
| 15 days | O | | |
| 16 days | O – | OO | OOω̃ – |
| 17 days | OO – | OOOO | |

This experiment confirms the relative insusceptibility of 12-day embryos though it should be noted that some of these would probably have shown virus multiplication if they had been left for 2 or 3 further days' incubation. All the embryo lungs tested from eggs with positive amniotic fluids showed the presence of virus without any clear indication that more was produced at one age than at another. The geometric mean titres of 14, 16 and 17 day lungs were 340, 220 and 140, but these differences are not significant.

C. *The Character of Virus Obtained after 1–6 Days Growth in Chick Embryos Inoculated Amniotically.*

Thirty eggs were inoculated at 13 days' incubation into the amniotic cavity with 0·1 c.c. of a 1: 1,000 dilution of lung emulsion 93065. This dilution was known from previous experiments to produce O type infections in all embryos tested at 3 days.

The eggs were opened at 1, 2, 3, 4, 5 and 6 days after inoculation, the amniotic fluid was collected and the lungs removed from the embryo. Virus titrations of fluid and lung were made by agglutination of fowl and guinea-pig cells.

TABLE 8.

*Changes in virus produced at various times after amniotic inoculation.*

| Time after inoculation. | Volume of A.F. | F/G A.F. | F/G Lung. | Type. | Geometric mean titre guinea-pig cells. A.F. | Lung. |
|---|---|---|---|---|---|---|
| 1 day | 1·1 c.c. | – – – | – – – | – – – | 0 | 0 |
| 2 days | 2·0 c.c. | – – 0/25 0/50 0/25 | – – 0/120 0/160 0/160 | – – OOO | 18 | 36 |
| 3 days | 1·5 c.c. | 60/180 0/280 0/140 20/60 0/200 | 80/240 0/280 5/620 0/30 8/200 | OOOO ω̃ | 150 | 200 |
| 4 days | 0·9 c.c. | 20/110 18/160 0/100 0/200 0/240 0/200 | 0/8 0/30 0/0 0/0 30/160 | OOOOOO | 160 | 15 |
| 5 days | 0·12 c.c. | 0/140 320/800 12/400 10/600 33/80 | 0/0 25/60 0/20 0/0 | O ω̃ OOD | 300 | 15 |
| 6 days | 0·07 c.e. | 30/50* 50/120* 10/40* 25/120* | 0/0 10/15 0/0 0/0 | ω̃ω̃ω̃ω̃ | 70* | 5 |

* Owing to the minute amount of fluid in the amniotic cavity these estimations were made on washings obtained by introducing about 1 ml. of saline into the cavity.

This experiment indicates clearly the rôle of the embryo lung in producing virus but it does not provide any evidence as to whether infection of the lung precedes general infection of the amniotic cavity. During the 1st, 2nd and 3rd day after inoculation each embryo showed either no demonstrable virus in lung and amniotic fluid or virus of the same type in each. Using the arbitrary convention of comparing amniotic fluid with the supernatant fluid from an emulsion of the embryo lungs in 1 ml. of saline, the lung titre is higher on the second and third days and then drops precipitately, most lungs showing no agglutination at all. This fall can be correlated with the histological changes described previously (Burnet, 1940). When the whole of the respiratory epithelium of parabronchi and the developing respiratory epithelium has been destroyed by the virus, as occurs within 3 or 4 days, it is natural that no further production of virus can be demonstrated.

The characteristic changes in the volume of amniotic fluid from the 14th to the 19th day of incubation are shown in this series. Most of the influx of albumin into the amniotic cavity takes place after the 14th day and the simplest explanation of our former finding that an O infection increases while a D infection greatly decreases the amount of amniotic fluid probably depends on this phenomenon. With a D infection the entrance of albumin is in some way prevented, with the milder O infection the normal sequence results but the albuminous fluid is apparently not so rapidly swallowed by the infected chick as by the normal. In the present series there was no evidence of excess mortality due to the infection and in the last 2 days of the experiment the amniotic cavity was almost as free of fluid as in normal embryos of the same age.

The most interesting result of the experiment is the change in the character of the virus which takes place after the 4th day. Of the embryos containing virus demonstrable by haemagglutination on the 2nd, 3rd and 4th days 13/14 showed O type reactions, on the 5th day there were 2/5 $\tilde{\omega}$ reactions and on the 6th day all four surviving embryos gave $\tilde{\omega}$ type reactions with their amniotic fluids.

The increasing proportion of $\delta$ or D type virus present in the fluids as the infected embryos age can also be demonstrated by determining the titre to which the fluid will infect the allantoic cavity. This can be taken as a measure of the D content. With O and $\tilde{\omega}$ fluids the guinea-pig cell titre can be taken as a measure of the amount of O virus present and in this experiment the D titre expressed logarithmically was compared with an O titre calculated on the semiarbitrary basis that a guinea-pig haemagglutination titre of 40 was given the logarithmic value 7·0, one of 400 a value of 8·0 while other titres were adjusted to the same scale.

TABLE 9.

*Content of O and D virus in fluids and lung emulsions from representative material taken 2, 3, 5 and 6 days after inoculation of O virus.*

| Period since inoculation. | Amniotic fluids. F/G. | Equivalent O titre. | D titre. | O–D. | Lung emulsion. F/G. | Equivalent O titre. | D titre. | O–D. |
|---|---|---|---|---|---|---|---|---|
| 2 days O | 0/35 | 7·0 | 3·5 | 3·5 | 0/160 | 7·7 | 4·0 | 3·7 |
| 2 days O | 0/50 | 7·2 | 2·7 | 4·5 | 0/160 | 7·7 | 4·0 | 3·7 |
| 3 days O | 0/280 | 7·8 | 5·0 | 2·8 | 0/280 | 7·8 | 4·8 | 3·0 |
| 3 days O | 0/140 | 7·5 | 4·7 | 2·8 | 5/640 | 8·2 | 4·5 | 3·7 |
| 3 days $\tilde{\omega}$ | 60/180 | 7·8 | 6·5 | 1·3 | 80/240 | 7·8 | 5·0 | 2·8 |
| 5 days O | 12/400 | 8·0 | 6± | 2± | 0/20 | 6·7 | 5·4 | 1·3 |
| 6 days $\tilde{\omega}$ | 30/50 | 7·2 | 5·5 | 1·7 | 0/0 | <6 | 4+ | <2 |
| 6 days $\tilde{\omega}$ | 50/120 | 7·5 | 6·7 | 0·8 | 10/15 | 6·5 | 5·5 | 1·0 |

O titres deduced from guinea-pig cell titre, D titres by result of allantoic titration in chick embryos. All shown logarithmically.

The figures for the D titres are only accurate to 1 logarithmic unit so that the overall features of the Table only are significant. It shows clearly, however, that there is a progressive approximation of the allantoic titre to that corresponding to the O content of the material.

D. *Production of Virus in Embryo Lung.*

Our first work on amniotic infections with influenza virus was based on the hypothesis that infection would primarily involve the embryo lung. All subsequent work has shown the high susceptibility of the lung but it has been very difficult to prove or disprove the primary character of the lung infection.

It is almost the rule to find that amniotic fluid and lung emulsion both contain virus in the same phase or are both free of virus. From 127 records of F/G titrations of both amniotic fluid and lung, 105 pairs showed correspondence and 12 showed only minor differences in the type of intermediate present. Of the remaining ten 5 showed virus with typical O character in the lung with $\tilde{\omega}$ virus in the amniotic fluid, while in one instance the opposite held. Two pairs gave detectable virus (O) in the lungs but not in the amniotic fluid while two gave positive findings in the amniotic fluid but not in the lungs.

In two instances we obtained rather clear evidence of primary lung infection. An embryo 93065 inoculated with O virus $10^{-6}$ was examined at 44 hours. The amniotic fluid failed to agglutinate guinea-pig cells but a small amount of fluid removed from the trachea and diluted approximately 1: 20 gave nearly complete agglutination. The lung emulsion from this embryo agglutinated at 1: 10 and infected embryos inoculated amniotically to a dilution of 1: $10^6$. This emulsion contained the highest proportion of O virus of any unabsorbed preparation with which we have worked and was used for the experiment shown in Table 7.

In a second similar instance an embryo inoculated at 14 days' inoculation and examined 44 hours later gave amniotic fluid showing weak agglutination of guinea-pig cells at 1: 10 only, a small drop of tracheal fluid giving complete agglutination at approximately 1: 20, and lung emulsion of F/G titre 1: 80. It was particularly interesting, however, to find the next embryo of the same series, identically treated, showing the following titres: Amniotic fluid 0/20. Tracheal fluid 0/trace. Lung 0/0.

The influence of age of the embryo on the results obtained after amniotic inoculation is best interpreted on the view that O infection is most readily initiated in the respiratory epithelium of the lung. To ensure this it is necessary both that there should be an adequate amount of respiratory epithelium and that a mechanism should be available to bring the inoculated virus into contact with it. About the 14th day of incubation two processes become active which favour lung infection. The parabronchi then begin to bud actively to produce the lung capillaries with a great increase in the available surface of respiratory epithelium. At the 14th or 15th day the sero-amniotic connection opens allowing albumin to pass into the amnion where is is swallowed and digested by the embryo. The swallowing movements probably play a large part in allowing fluid to move up and down the trachea and so facilitate infection.

It is impossible, however, to conclude that O virus multiplies only in the lung. It can multiply in other parts of the various surfaces bathed by amniotic fluid and even under exceptional circumstances within the allantoic cavity but it is in accord with all the facts to state the situations, embryo lung, amniotic cavity, allantoic cavity, provide successive environments in which O virus multiplies with increasing difficulty and where any appearance of D virus more rapidly overgrows the O phase.

E. *Absorption-Elution Experiments with O and Intermediate Type Virus.*

When O virus is treated with guinea-pig cells according to the methods used by Hirst absorption and elution can be demonstrated in typical fashion. The eluted virus has the same general character as the original.

Experiments with fowl cells have given more interesting results. In the first place totally different results are obtained when the same O or $\tilde{\omega}$ virus is used (a) in the form of amniotic fluid, (b) as embryo lung emulsion. The amniotic fluid virus is completely absorbed by a standard dose of washed fowl cells in the cold and about half of the virus can be eluted from the cells by exposure to 37° C. for 2–3 hours. With an embryo lung emulsion there is no demonstrable absorption of an O virus and no virus is obtained from the cells by elution. The difference is due to some constituent of the amniotic fluid a mixture of lung virus with normal amniotic fluid showing the same type of reaction as amniotic fluid virus.

The embryonic lung preparations can therefore be regarded as showing a more "natural" type of behaviour and analysis of intermediate type virus has been attempted with such material. The following experiment with a pool of $\tilde{\omega}$ type lung virus is typical.

One ml. of the virus with an original titre of F/G 60/240 was treated in the cold with 0·1 ml. of washed packed fowl cells for 30 minutes. It was then spun and the supernatant fluid preserved. The cells were washed once with cold saline and the deposit emulsified in 1 ml. of warm saline and left for 2½ hours to elute at 37° C. Both the absorbed fluid and the eluate were again subjected to the same process. The F/G titres obtained can be shown as follows:

Original 60/240 → Absorbed 0/60 → Absorbed 0/50*; Eluate —
Original 60/240 → Eluate 50/120 → Absorbed 0/15; Eluate 50/90

A $\delta$ type pool treated similarly gave the following F/G titres:

Original 240/400 → Absorbed 0/15 → Absorbed 0/5; Eluate 0/0
Original 240/400 → Eluate 200/320 → Absorbed 0/15; Eluate 160/160

For comparison an O virus gave the following:

Original 0/480 → Absorbed 0/480 → Absorbed 0/480; Eluate 0/30
Original 0/480 → Eluate 0/40 → Absorbed 0/20; Eluate 0/20

One may conclude that at least a considerable fraction of O virus is not capable of being absorbed by fowl cells and that from intermediate types one can separate on the one hand virus with the *in vitro* characteristics of O and on the other (by elution) virus which gives reactions nearer to D than the original.

Evidence that O virus can actually be separated from a preparation giving ω̃ reactions was obtained by inoculating high dilutions of the twice absorbed fluid marked * 0/50 derived from an ω̃ lung with F/G 60/240. The original fluid was not titrated but all our experience makes it certain that no O fluids would have been obtained in such a titration. The twice absorbed fluid gave the results shown.

TABLE 10.

*Results of inoculation of virus absorbed with fowl red cells.*

| | Dilution. | | | | | |
|---|---|---|---|---|---|---|
| | $10^{-5}$. | | $10^{-6}$. | | | |
| Amniotic fluid | 0/25 | 60/200 | 0/280 | 0/100 | 0/160 | — |
| Lung | 10/60 | 120/640 | 0/640 | 0/320 | 0/480 | — |

This is a perfectly typical result of an O titration indicating that the *in vitro* results provided a true indication of a sorting out of different types of virus from the original mixture.

A similar experiment was carried out on an artificial mixture of 9 parts of O virus (F/G 0/320) with 1 part of δ virus (F/G 640/1280). The mixture gave an ω̃ type agglutination F/G 80/500.

Using the same technique of absorption and elution the following *in vitro* results were obtained:

Original 80/500 → Absorbed 0/320 → Absorbed 0/280 (S2); —
Original 80/500 → Eluate 60/120 → Absorbed 0/5; Eluate 50/70 (E2)

Amniotic and allantoic titrations of the original mixture, the second supernatant S2 and the second eluate E2 gave the results shown in Table 11.

TABLE 11.

*Separation of O and δ virus by absorption and elution.*

| | Original. | | | S2. | | | E2. | | |
|---|---|---|---|---|---|---|---|---|---|
| Dilution. | Amniotic. | Allantoic. | | Amniotic. | Allantoic. | | Amniotic. | Allantoic. | |
| | | + | − | | + | − | | + | − |
| $10^{-6}$ | OOω̃ω̃ω̃−−− | 0 | 4 | | | | | 0 | 4 |
| $10^{-7}$ | ω̃δδδδ | 4 | 4 | O−−−−−−− | 1 | 3 | δδδδδδ | 2 | 6 |
| $10^{-8}$ | δδδδδδ | 8 | 0 | OOOO−−−− | 0 | 4 | δδδδδδ | 7 | 1 |
| $10^{-9}$ | | 4 | 0 | δδ− | 2 | 2 | | 4 | 1 |
| $10^{-10}$ | | | | | 4 | 0 | | | |

The type of virus obtained is shown under amniotic titration. The number of fluids positive or negative by guinea-pig cell agglutination under allantoic titration.

## DISCUSSION.

These findings fully confirm the general picture of the O–D transformation developed in earlier papers but they indicate the existence of a considerable variety of possible intermediate forms. A typical O fluid or lung emulsion which gives no agglutination of fowl cells or at most a trace of agglutination at a dilution of 1 in 5 or less and a guinea-pig cell titre of 80 or more is always derived from an inoculum which fails to multiply in the allantoic cavity and in suitable embryos (of physiological age 13–14 days) will always give higher titres by amniotic than by allantoic titration. A typical D fluid with an F/G ratio of 1 or higher will always multiply to full titre in the allantoic cavity. The intermediate type fluids, however, do not always behave as if an increasing F/G ratio is directly correlated with

increasing ability to multiply in the allantoic cavity. On any reasonable hypothesis all the O and intermediate type fluids which one can obtain are mixtures, the F/G titre and the type of result obtained by subinoculating limiting dilutions merely defining the dominant component of the mixture. For this reason we have found it impossible to decide rigorously whether there are a small number (say 2) of intermediate genotypes or whether there is an almost unlimited number of unstable genetic configurations which can be derived from the O phase. The simplest interpretation we can offer is that virus preparations of the O-ω–δ–D series represent populations of virus particles which differ in respect of (a) the effectiveness with which they are absorbed to and can agglutinate fowl cells and (b) the readiness with which they can initiate infection in the allantoic cavity. These differences may in part represent non-genetic variability within the genotype but many of them are certainly inheritable. In general the two characters are correlated but this is not always the case, e.g., the allantoic fluid virus described above with F/G titre 0/20.

The nature of the virus material obtained by the multiplication of a given inoculum in the chick embryo depends (1) on the population of virus particles inoculated, (2) on the time at which any mutant descendant particles appear and on their character and frequency of occurrence and (3) on the relative rates of multiplication of the different types of virus particle present under the conditions provided in the embryo.

Limited experiments typified by that shown in Table 6 suggest that the age and state of nutrition of the embryo as well as the route of inoculation play a large part in determining the relative rates of proliferation of the different types of particle and hence the gross character of the material eventually harvested.

The complexity of the conditions makes it impossible to devise quantitative experiments (along the lines of those of Luria and Delbruck, 1943) to show whether or not the variant types appear as mutants with a determinable frequency. The impression obtained from the whole investigation was that the findings were fully compatible with a mutation hypothesis and that the lack of complete reproducibility in the results was in part a necessary consequence of the mutation process (cf. Luria and Delbruck's findings) and in part due to individual differences especially in physiological age amongst the chick embryos used.

For the past 10 years all our experimental eggs have been obtained from the same poultry farm. In order to eliminate the possibility that our results were only obtainable with one strain of embryos an experiment was set up with White Leghorn eggs from an entirely distinct source:—a "mammoth" poultry farm where the general regime differed considerably from that in the relatively small establishment of our regular supplier. These eggs were received as 11-day embryos and were incubated in the laboratory for varying times before amniotic inoculation with O virus. The results indicated that these embryos behaved like our standard ones except that their physiological age was about a day less, i.e., satisfactory results were obtained with 14-day and older embryos but not with 13-day embryos.

From personal communications we gather that other workers have found difficulty in maintaining the O character on passage. Our current experience suggests that this can be done with certainty if the following precautions are observed. (A) The inoculum is prepared from an embryo lung emulsion (not from amniotic fluid) by absorption with fowl cells according to the technique mentioned in the text. The initial emulsion should show an F : G haemagglutination ratio of 1 : 10 or less while the absorbed fluid should show no fowl cell agglutination whatever. (B) Dilutions from this in normal horse serum saline, $10^{-4}$, $10^{-5}$ and $10^{-6}$ are inoculated into the amniotic cavity of embryos which have reached the full physiological development of a 13–14 day embryo. (C) The lungs are harvested after not more than 72 hours' experimental incubation at 35° C.

SUMMARY.

An influenza virus A strain (BEL) has been maintained in the O phase through 24 amniotic passages. No change has occurred in the essential characteristics of low F/G ratio and inability to multiply in the allantoic cavity.

Multiplication of O virus is more rapid in 13 or 14-day than in 11 or 12-day embryos. This may be related to the active development of respiratory epithelium in the lung at this period.

In a large proportion of embryos inoculated amniotically with pure O phase virus (i.e. at high dilutions) there is a transient high concentration of O virus in the lung. This provides a valuable source of material free of the excess albumin present in most positive amniotic fluids.

In embryos inoculated amniotically at 13 days' incubation with pure O virus the amniotic fluid and lung removed after 2 or 3 days' subsequent incubation may contain more than 1,000 times as much O as D virus but the proportion of D or intermediate type virus rises with further incubation and no typical O materials are obtained after the 5th day.

Fluids or lung emulsions can be obtained which are intermediate in character between typical O and D preparations. Two types $\tilde{\omega}$ and $\delta$ are defined but it has not been possible to obtain a clear interpretation of the significance of these intermediate phases.

Embryo-lung virus in the O phase is not absorbed by fowl cells. This allows a method of partial purification of O virus.

Some natural lung emulsions giving $\tilde{\omega}$ type reactions and artificial mixtures of O and $\delta$ lung emulsions also showing $\tilde{\omega}$ type F/G reactions, can be partially separated into O and $\delta$ components by absorption and elution with fowl cells.

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# A METHOD FOR THE ISOLATION OF INFLUENZA VIRUS FROM THROAT WASHINGS WITHOUT FILTRATION

by F. M. BURNET AND J. D. STONE[1]

(From the Walter and Eliza Hall Institute, Melbourne).

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Several workers have considered methods for the isolation of influenza virus by treating throat washings with suitable antibacterial agents. Krueger *et al.* used zephiran 1:20,000, Sigurdsson (1944) showed that sodium sulphadiazine had no inhibitory effect on the growth of influenza virus in the allantoic cavity. Hirst in December 1943 (personal communication) was experimenting with penicillin treatment of throat washings before inoculating them into chick embryos by the amniotic route.

Owing to the absence of epidemic influenza in Melbourne during the winter of 1944 no successful primary isolations of virus have been made by methods of this type. A convenient method has been developed, however, which will allow the quantitative recovery of small amounts of "O" influenza virus A, added to unfiltered throat washings of normal persons. It has also been applied to a number of washings from respiratory infections (not influenza A or B) with negative results. In view of the probable usefulness of the method for primary isolation of influenza virus a brief account is submitted although it is recognized that until it has been applied to natural epidemic material some doubt as to its effectiveness must remain.

The technique is as follows: The patient is given 10 ml. of saline to gargle for one minute. Five ml. of nutrient broth are added to the garglings and sufficient penicillin solution to give a concentration of 100 oxford units per ml. This is filtered through sterile filter paper to remove gross particles.

Amniotic inoculations are made in 13-day chick embryos according to the technique developed in this laboratory but in addition to 0·1 of the throat washing an equal volume of 5 p.c. sodium sulphadiazine solution is also inoculated into the amniotic cavity. Our practice is to have two capillary pipettes with bevelled points, one being used for the sodium sulphadiazine, the other for the infective inoculum. The drug is given first and is followed immediately by the inoculum from the other pipette. The eggs are sealed as usual and examined after 4 or 5 days' incubation.

In five experiments in which a 1:100 dilution of O virus was made respectively in penicillin-treated throat washings and in normal horse-serum saline, the results obtained in titrating the two series were not significantly different. In the last of these experiments all dilutions of the first series were made in the penicillin-throat washing and inoculated with sodium sulphadiazine, while the second series of dilutions in normal horse-serum saline were inoculated in the normal way.

TABLE 1.

*Comparative titration of O influenza virus 94250 in penicillin-treated throat garglings and in normal horse-serum saline.*

| Final dilution of virus. | Throat washings. | Normal horse-serum saline. |
|---|---|---|
| $10^{-7}$ | OOO | O– – |
| $10^{-8}$ | $\bar{\omega}\bar{\omega}\delta\delta$ | OO $\bar{\omega}\bar{\omega}$ |
| $10^{-9}$ | $\bar{\omega}\bar{\omega}\delta\delta$ | $\bar{\omega}\bar{\omega}\delta\delta$ |

O, $\bar{\omega}$, $\delta$, recovery of virus in phase shown; –, no evidence of infection.

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The results shown in Table 1 are expressed according to the phase of the virus recovered in the amniotic fluid. The terms $\tilde{\omega}$ and $\delta$ used for intermediate types are defined and discussed in a recent paper (Burnet and Stone, 1945). In previous work $\tilde{\omega}$ would have been classed as O, $\delta$ as D.

Cultures from the amniotic fluids have been consistently negative and on a number of occasions subinoculations of positive or negative fluids to other embryos have given no evidence of bacterial contamination.

Attempts to use a similar method for yolk sac inoculations have been unsuccessful, bacterial infection almost always becoming evident. Apparently the large volume of fluid in the yolk sac at 6 days' incubation and the ease with which bacteria can multiply in this fluid are responsible for the failure of the bacteriostatic agents to control the infection. A more direct application of the method has been to the reisolation of virus from the noses of ferrets inoculated intranasally with O virus. In the course of an experiment on the possibility of immunizing ferrets with concentrated vaccine given subcutaneously, nasal washings were obtained from 4 animals at a time when they were showing a significant temperature rise 2 or 3 days after inoculation with 0·05 ml. of a 1: 250 dilution of O virus. Two of the ferrets were normal animals, 197 and 198, two had been immunized, 194 and 195.

The washings were obtained by a technique described by Burnet and Bull (1943) in which the lightly anaesthetized ferret is allowed to recover while being held vertically with its nostrils dipping into 15 ml. of broth in a tilted petri dish. Most ferrets in this situation breathe in through the mouth and out through the nose and after a minute's bubbling a considerable amount of infective fluid passes into the broth. This was then treated precisely as a human throat filtrate. In this experiment the washings from the two control ferrets 197 and 198 were pooled and divided, half being filtered through a gradocol membrane while the other half was treated with penicillin and inoculated with sodium sulphadiazine. Both washings from the previously immunized ferrets were used unfiltered with the bacteriostatic agents. Table 2 gives the results obtained.

TABLE 2.

*Isolation of influenza virus from nasal washings of experimentally infected ferrets.*

| Ferret number and status. | Method. | Result of amniotic inoculation. Undiluted. | $10^{-1}$. | $10^{-2}$. | $10^{-3}$. | $10^{-4}$. |
|---|---|---|---|---|---|---|
| F197–8 (normal) | Filtration | $\tilde{\omega}\tilde{\omega}\tilde{\omega}$O | $\tilde{\omega}\tilde{\omega}$Ox | OO – – | | |
| | P. and S.D. | $\underline{\tilde{\omega}\tilde{\omega}}$xx | $\underline{\tilde{\omega}}$OOO | OOOx | OOOx | O – – x |
| F194 (immunized) | P. and S.D. | $\underline{\tilde{\omega}\tilde{\omega}\tilde{\omega}\tilde{\omega}}$ | | OO – – | | |
| F195 (immunized) | P. and S.D. | $\underline{\tilde{\omega}\tilde{\omega}\tilde{\omega}}$O | | $\tilde{\omega}$OOO | OO – – | O – – – |

$\tilde{\omega}$, O virus present of phase incidated; –, no agglutination by amniotic fluid; x, death of embryo before 72 hours. Symbols underlined indicate that fluid contaminated as judged by culture of a loopful on a blood agar plate. P. and S.D., Penicillin and sodium sulphadiazine.

As might be expected the ferret washings contained sufficient Gram negative bacilli to contaminate most of the embryos inoculated with undiluted material but the bacteriostatic agents used were sufficiently effective to allow all but two of the embryos to survive 3 days and to allow free multiplication of influenza virus. It will be seen that in the direct comparison between the two methods the filtered material is possibly of lower titre than the unfiltered. Both show the typical behaviour of an O type virus giving O fluids only at the higher dilutions. This provides evidence for the free proliferation of O virus in the respiratory tract of the ferret.

## DISCUSSION.

Although we have had no opportunity to apply the method to the actual problem of virus isolation we feel that these experiments suggest that the most satisfactory procedure for a comprehensive attempt to isolate influenza viruses is as follows:

1. Obtain garglings from patient, preferably first thing on waking in the morning. Preserve the fluid on ice till it can be dealt with in the laboratory.

2. (a) Infect one or two ferrets intranasally with unfiltered material.
   (b) Wash out nasal cavity when the temperature rises or at the third day and titrate washings, as described above, in amniotic cavity.
   (c) Bleed ferrets for serological tests 14 days after inoculation of garglings.
3. (a) Treat garglings as described above and inoculate 6, 13 or 14–day embryos amniotically.
   (b) Test half the embryos at 3 days and half at 5 days for guinea-pig cell agglutination by amniotic fluid and by lung emulsion.
   (c) If all results appear negative, pool all amniotic fluids (after test for bacterial sterility) of the first passage and subinoculate amniotically into 6 eggs. If these embryos are negative at 5 days the material can be regarded as containing no viable influenza virus.

The advantages of the method are (1) that it makes full use of the susceptibility of the ferret since the fact of infection may be established either by symptoms, by reisolation of virus or by the recognition of an antibody response. (2) The use of two different hosts (ferret and chick embryo) for attempted primary initiation of infection will increase the likelihood of obtaining infection in one or the other with strains on the borderline of pathogenicity. (3) From either host the virus is obtainable in the unmodified O form and can be used at once or within one embryo passage for *in vitro* studies.

Finally, it may be mentioned that the technique described should be applicable in principle to the isolation of any micro-organism pathogenic for the chick embryo and unaffected by the two bacteriostatic agents used.

## SUMMARY.

By the use of penicillin and sodium sulphadiazine it is possible to isolate influenza virus in the O form from unfiltered human throat washings in which it is present.

The same method has allowed the successful isolation of virus from the nasal secretion of experimentally infected ferrets.

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